STUDIES ON NEURAMINIDASES PRODUCED BY ANAEROBIC BACTERIA AND THEIR POSSIBLE ROLE IN PATHOGENICITY

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STATEMENT

This thesis and the studies reported in it are my own work, performed with advice and guidance from Professor J.G. Collee and with the invaluable technical support of Robert Brown and his assistants in the Microbial Pathogenicity Research Laboratory, Department of Bacteriology, University of Edinburgh.

The preparation of the human glycoprotein substrate (see Section Ia) was undertaken by Dr J.K. Smith, Scottish National Blood Transfusion Service, Protein Fractionation Centre, Ellen`s Glen Road, Edinburgh EH17 7QT; the extent of Dr Smith`s contribution is made clear in the text.

Parts of these studies have already been published; the relevant papers are included in the Appendix, with a note on authorship.

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"Nel mezzo del cammin di nostra vita
mi ritrovai per una selva oscura"

Dante Alighieri

"If at first you don't succeed -
redefine success"

Graffito in the Erskine
Medical Library, 1983

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ABSTRACT

These studies were designed to investigate the production of neuraminidase by a wide range of species of strictly anaerobic bacteria and to explore the possibility that the enzyme may play a part in the pathogenic mechanisms of these organisms.

A glycoprotein fraction was prepared from pooled human plasma for use as substrate in assays for neuraminidase. Sensitive and reliable procedures were developed for characterisation of neuraminidase production by Clostridium perfringens and Bacteroides fragilis, and these studies were extended to a survey of well characterised strains of other species in these genera. There was variation amongst strains of C. perfringens type A; many heat-resistant food-poisoning strains did not produce the enzyme whereas most haemolytic, heat-sensitive strains did. The results were consistent for all strains of the other clostridial and bacteroides species examined and it is suggested that tests for neuraminidase production should be a valuable addition to currently used biochemical tests in certain areas of taxonomic studies in both genera.

The ability of various clostridia to produce experimental myonecrosis was tested in guinea pigs. Strains classed as neuraminidase-positive in vitro also produced the enzyme in vivo; neuraminidase-negative strains did not. Pathogenic clostridia produced extensive myonecrosis and death in 24-48 h; virulence was assessed by the challenge dose required to produce fatal infection or by the severity of local muscle infection in surviving animals. Although a number of pathogenic clostridia produce neuraminidase,

there was no clear-cut correlation between virulence and ability to produce the enzyme. Large amounts of neuraminidase were produced in the tissues by virulent strains of C. perfringens and C. septicum but none by the equally pathogenic but neuraminidase-negative C. novyi; at least one neuraminidase-negative strain of C. perfringens was able to produce fatal infection.

Various equine and rabbit antisera were assessed for their ability to protect animals challenged with a virulent strain of C. perfringens; the protective effect was found to correlate with the content of anti-α-toxin. C. perfringens neuraminidase proved to be a poor antigen and it was difficult to produce sera with high levels of neutralising activity; however, there was no evidence that anti-neuraminidase contributed to the protective effect of the antisera tested.

The occurrence and roles of neuraminidase and its substrates are reviewed, and various ways in which neuraminidase produced during infection might contribute to tissue damage are discussed. The pathogenesis of C. perfringens gas gangrene remains incompletely understood and a number of features of the disease are not adequately explained by theories that attribute the major role to α-toxin. Neuraminidase is one of a collection of digestive enzymes that are thought to be of value to commensal or saprophytic C. perfringens strains and it is probable that it has a similar role during infection; however, there is no evidence that neuraminidase is an important virulence factor for the organism.

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INTRODUCTORY REVIEW

The enzyme neuraminidase (sialidase; N-acyl neuraminosyl glycohydrolase; EC 3.2.1.18) is widely distributed in nature, being found in viruses, bacteria, protozoa and vertebrate tissues, though not in plants. The enzyme acts to release sialic acids (neuraminic acid derivatives) from carbohydrates, glycoproteins and glycolipids that have a variety of important roles in various animal tissues. Sialic acids are thought to contribute to the specific biological properties of many sialoglycoconjugates, and neuraminidases have been widely used as tools in studies on their activities. Mammalian neuraminidase is predominantly packaged as a lysosomal enzyme and is thought to be involved in the controlled degradation of sialoglycoconjugates in normal animals; this may contribute to the regulatory processes that determine the activity and turnover of various glycoconjugates and cells.

The enzyme is produced by a number of important pathogenic micro-organisms and it has been suggested that it may play a role in pathogenesis of infections produced by some of these organisms. The enzyme is not directly toxic to experimental animals but it has been shown to be able to affect a number of important properties of a variety of cells and glycoproteins *in vitro* and it seems possible that the presence of exogenous enzyme during infection might contribute to the pathogenic process.

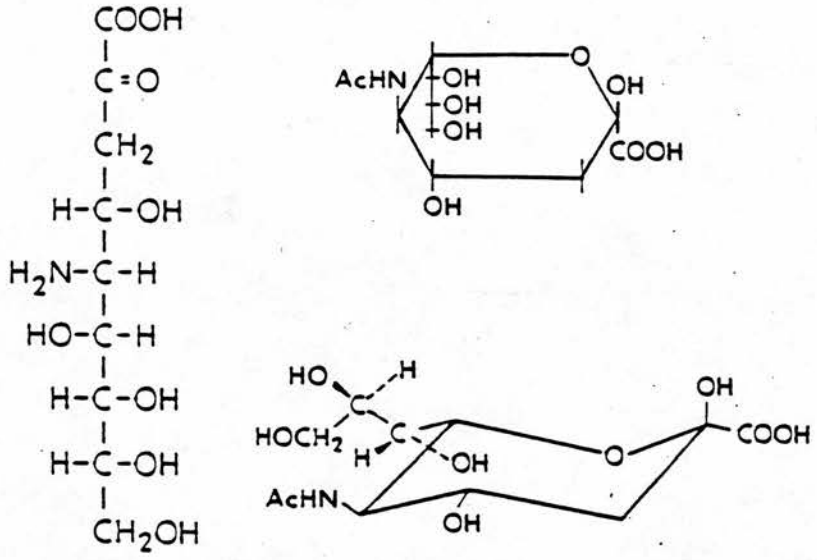
The Introduction to this report reviews the occurrence and roles of neuraminidase and its substrates and the possible role of the enzyme in a variety of infections. The experimental work

reported thereafter was undertaken in order to study further the production of the enzyme by a range of anaerobic bacteria of clinical interest and to assess the possible contribution of neuraminidase to the pathogenesis of clostridial myonecrosis.

Discovery and chemistry of sialic acids

The early history of the discovery of sialic acids was reviewed by Gottschalk (1960); more recent accounts of the chemistry and biological roles of these amino sugars were given by Blix & Jeanloz (1969), Tuppy & Gottschalk (1972), Ledeen & Yu (1976), Corfield & Schauer (1982b) and Schauer (1982). The accepted nomenclature of the sialic acids was proposed by Blix, Gottschalk & Klenk (1957). The term neuraminic acid is reserved for the unsubstituted parent compound whose structure is shown in fig. Intro/1(a); this compound is inherently unstable and has not been isolated. The naturally occurring N-acylated and O-acylated derivatives are known collectively as sialic acids. The commonest form is N-acetyl neuraminic acid (NANA) whose structure is shown in fig. Intro/1(b); this is the precursor from which other sialic acids are synthesised. Many species of animals, though not man, have a proportion of sialic acids with an N-glycolyl group in place of the N-acetyl group at position 5. Diacyl compounds also occur, with the molecule further substituted with an O-acyl group in position 4, 7, 8 or 9; triacyl derivatives are also encountered (Ledeen & Yu, 1976; Schauer, 1982).

The sialic acids occur predominantly in the carbohydrate portion of glycoproteins or glycolipids and in various oligo- and



(a) (b)

Fig. Intro/1 - (a) Structure of neuraminic acid in the open-chain (keto) form; (b) representations of structure of N-acetyl neuraminic acid (NANA) in the pyranose form (from Ledeen & Yu, 1976).

poly-saccharides; only small quantities are found in the free state. The occurrence of the various sialic acids was reviewed by Ng & Dain (1976) and Schauer (1982). Human tissues contain only N-acetyl sialic acids - predominantly NANA itself - but many animal species also have N-glycolyl and N,O-diacyl derivatives (Ledeen & Yu, 1976). Sialic acids occur linked with lactose in various forms of sialyl-lactose, oligosaccharides which are found in high concentrations in mammalian colostrum. Around 80% of the NAN-lactose in bovine colostrum is in the N-acetylneuramin ($\alpha 2 \rightarrow 3$) linkage but the ($\alpha 2 \rightarrow 6$) linkage also occurs (Schneir & Rafelson, 1966). Colominic acid, a polymer of ($\alpha 2 \rightarrow 8$) linked NANA, is produced by strains of Escherichia coli type K1 and Neisseria meningitidis group B (Barry, 1958; Kasper et al., 1973; Robbins et al., 1974); it has been suggested that this unusual capsular material may be a virulence factor in the production of neonatal meningitis by these otherwise dissimilar bacteria (Orskov, 1978; Wilfert, 1978). Group-B streptococci, which may cause neonatal meningitis, also contain sialic acid in their surface heteropolysaccharide (Baker & Kasper, 1976).

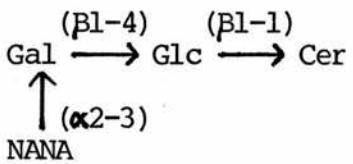
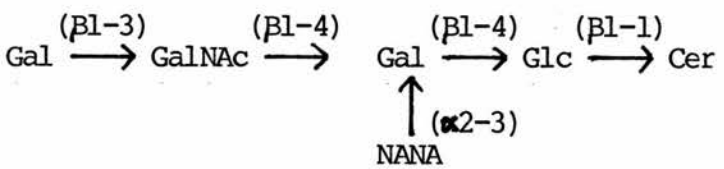
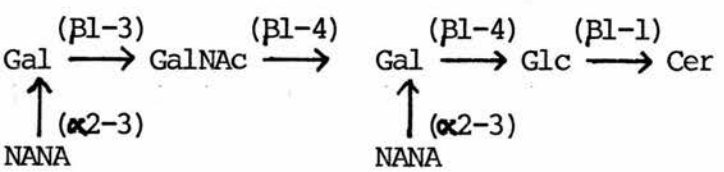
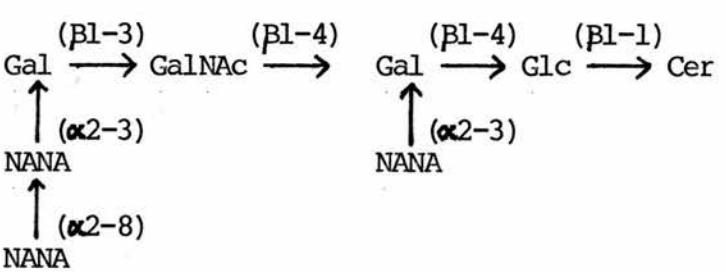
NANA is a common constituent of mammalian glycoproteins and was first purified by Blix in 1936 during studies on bovine submaxillary mucin (see Gottschalk, 1960); the name sialic acid derives from its detection in salivary gland material. The sialic acids in glycoproteins occur as terminal sugars bound predominantly by ($2 \rightarrow 3$) or ($2 \rightarrow 6$) linkages to galactose or N-acetyl D-galactosamine, or occasionally to N-acetyl D-glucosamine; evidence for ($2 \rightarrow 4$) and ($2 \rightarrow 9$) linkages in glycoproteins has also been reported (Schauer, 1982). They also occur bound by ($2 \rightarrow 8$) linkage

to another sialic acid in compounds with a terminal disialyl group (Gottschalk & Drzeniek, 1972). Sialic acids in cell-membrane glycoproteins are major cell-surface constituents and appear to have important roles in cell behaviour and interactions.

Sialic acids are also present in glycolipids isolated from cell membranes and brain tissue (see reviews by Brunngraber, 1979; Li & Li, 1982). Sialic-acid-containing glycosphingolipids are called gangliosides; some 15 inter-related gangliosides have been isolated from brain and other tissues (Fishman & Brady, 1976b). The term neuraminic acid was first used by Klenk in 1941 for a substance isolated from human brain, and eventually shown to be similar to that found by Blix in submaxillary mucin (see Gottschalk, 1960). Human gangliosides contain only N-acetyl derivatives whereas those from cell membranes of other mammalian species may also contain N-glycolyl sialic acids. Virtually all the ganglioside sialic acid in mammalian brain is NANA, regardless of species (Ledeen & Yu, 1976). Gangliosides are classified by the composition of the oligosaccharide chains attached to the ceramide portion of the molecule (see Ng & Dain, 1976). The carbohydrate portion of gangliosides may contain up to four neutral sugars and one, two or three NANA molecules (though minor constituents have been detected with up to five). NANA usually occurs as the terminal sugar, bound by (2 $\rightarrow$ 3) linkage to galactose; occasionally it occurs as a terminal disialyl (2$\rightarrow$8) group. The structure is usually branched, with a further NANA linked (2$\rightarrow$3) to an internal galactose (see table Intro/I). Sequential removal of sialic acid residues from gangliosides by neuraminidase leads to

TABLE Intro/I

Oligosaccharide structure of four gangliosides from vertebrate brain

Ganglioside	Structure of oligosaccharide[a]
GM3	
GM1	
GD1a	
GT1a	

[a] From Ng & Dain (1976).

their interconversion as the terminal molecules are removed to leave monosialyl gangliosides (e.g. GM1 or GM2) with a terminal galactose residue but retaining the "internal" NANA; this usually cannot be removed until the carbohydrate chain has been further degraded (Suzuki, 1976).

The evolution of sialic acids has been discussed by Ng & Dain (1976), Corfield & Schauer (1982a) and Schauer (1982). They do not occur in plants, insects and primitive invertebrates, appearing first in higher invertebrates; conflicting reports of the finding of trace amounts of sialic acids in various lower species may be attributable to unrefined assay techniques or to presence of sialic acids in the diet or gut contents. The presence of sialic acids in viruses may be ascribed to their close links with higher host cells. Some bacteria, both Gram-positive and Gram-negative, contain sialic acids and have enzyme systems for their metabolism; it has been suggested that these occur mainly in pathogenic bacteria (Irani & Ganapathi, 1962) and that they too may have been acquired by contact or association with higher animals (Ng & Dain, 1976). Sialic-acid-containing gangliosides appear in the brain in fish and amphibia, and increasingly complex sialic acid metabolism is found in reptiles, birds and mammals. It is striking that the N-glycolyl derivatives are common throughout earlier species but have been discarded in man.

Discovery of neuraminidase

Neuraminidase (sialidase) was discovered in the course of observations on the interaction of influenza virus and red blood

cells (see Burnet, 1951; Gottschalk, 1960, 1966; Gottschalk & Bhargava, 1971). The ability of influenza viruses to adsorb to and agglutinate red cells was discovered independently in 1941 by Hirst and by McClelland & Hare (Hirst, 1942a). The virus can elute from the cells again; in the process it destroys the receptors on the red cell but leaves the virus able to repeat the cycle with fresh red cells. Hirst (1942b) ascribed this to an enzymic action of the virus at the surface of the cell. The modern view of influenza virus with separate haemagglutinin and neuraminidase subunits occurring as glycoprotein spikes projecting from the virus particle is reviewed in a later section below.

Burnet, McCrea & Stone (1946) observed that red cells treated with influenza virus not only lose their receptors for the virus but also have altered surface antigens such that they become polyagglutinable (panagglutinable), i.e. agglutinable by antibody present in normal human sera regardless of ABO specificity. Similar anomalies in blood-grouping tests had been described by Thomsen in 1926 and investigated further by Friedenreich (1928, 1930); it was concluded that a new antigen (T antigen) was produced on the cell surface by enzymic action in samples of red cells that had been contaminated by various bacteria, and that this could react with natural anti-T antibody present in most mammalian sera. Burnet and his colleagues, recognising the parallels, soon demonstrated the presence of enzymes that were able to induce polyagglutinability and to destroy influenza-virus receptors of red cells in culture filtrates of various bacteria, including

Clostridium perfringens (C. welchi) and Vibrio cholerae (Burnet et al., 1946). The Vibrio cholerae receptor-destroying enzyme (RDE) soon became a standard reagent in influenza virus studies.

The biochemical nature of the reaction was more conveniently studied after the realisation that serum glycoproteins that acted as inhibitors of influenza virus haemagglutination, e.g. the Francis inhibitor, were also substrates for the enzyme (Anderson, 1948) and the studies were soon extended to other readily available glycoproteins such as ovomucin (Gottschalk & Lind, 1949). These studies resulted in the characterisation of the split product as NANA and of the enzyme as an α-glycosidase that was named neuraminidase, or sialidase (Gottschalk, 1956, 1957).

The enzyme has since been isolated from other viruses (its presence has been used for classification of this whole group of viruses as myxoviruses) and it has also been found to be produced by many other bacteria (see below). Neuraminidases from different sources all release sialic acids from sialic-acid-containing substances but their properties may vary considerably, e.g. in MW, subunit structure, ion requirements, pH optima and specificity for $(2 \rightarrow 3)$, $(2 \rightarrow 6)$ or $(2 \rightarrow 8)$ linkages. Mammalian neuraminidase was first reported by Warren & Spearing (1960) but these enzymes have proved much harder to purify and characterise. The role of mammalian sialidases in normal metabolism of sialic-acid-containing compounds has been much studied. The initial observations on the effect of neuraminidase on red cells have been extended and the blood-group chemistry of the Thomsen-Friedenreich phenomenon is now understood in detail. Bacterial and viral neuraminidases have been widely used as tools in studies of carbohydrate structure and

function in other sites, e.g. in circulating glycoproteins and in surface components of white blood cells, tissue cells and tumour cells. A number of these areas are reviewed below.

BIOLOGICAL ROLES OF SIALIC ACIDS

One difficulty in ascribing particular functions to the sialic acid moieties in glycoproteins and other glycoconjugates is that the overall function of the carbohydrate portion itself is unknown. A general role for carbohydrate in export of glycoproteins from the synthesising cells has been suggested (Eylar, 1965) but appears unlikely, if only because of the wide variation in carbohydrate content - from a single residue per molecule to more than 40% by weight of certain glycoproteins - and the existence of some non-glycosylated extracellular proteins (Warren, Buck & Tuszynski, 1978). The enormous variety of spatial configurations that result from different combinations, conformations and linkages of a relatively small number of sugar residues suggests that the carbohydrate portions are concerned in conferring specificity of interaction and may more probably play a role in determining the destination of glycoproteins in the body than their export into the circulation (Winterburn & Phelps, 1972; Clamp, 1975).

No unified view of the biological role of sialic acids has emerged although it seems reasonable to assume that they are important in the regulation of a variety of biological processes because of their peripheral position in many glycoconjugates and their frequent external location in cell membranes (see Gottschalk, 1960; Rosenberg & Schengrund, 1976a; Jeanloz & Codington, 1976; Schauer, 1982). It is widely assumed that the biological properties of sialic acids are primarily attributable to the strong negative charge of the carboxyl group although direct evidence for

this is not always available. The influence of the different acyl substitutions observed in naturally occurring sialic acids is unknown; glycoproteins with equivalent functions in man and other mammalian species may be substituted with N-acetyl or N-glycolyl groups without obvious differences in biological function. Jeanloz & Codington (1976) have suggested that the polyhydroxylated side chain (C7-C9) may be of significance, since it is the result of a rather complex biosynthetic pathway and seems to be the only covalently bound, flexible, polyhydroxylated structure present on the surface of cells; there is, however, no direct evidence for its importance.

The negative charge on sialic acid residues may contribute to the conformation of glycoconjugates and may confer rigidity both on these molecules and on cell membranes in which they are concentrated (Montreuil, 1982; Schauer, 1982). Sialic acid contributes to the viscosity of glycoproteins in mucous secretions, which have a protective and lubricating role at mucous surfaces (Ahmad & McPhie, 1980). The presence of sialic acid residues gives resistance against proteolytic degradation in a variety of glycoproteins, predominantly those with a high sialic acid content (Hoskings, 1978). Sialic acid is characteristically the terminal sugar in an oligosaccharide; it may therefore be seen as a terminating signal for biosynthesis (Corfield & Schauer, 1982b). Catabolism of glycoconjugates is a stepwise process and the presence of terminal sialic acid residues protects the rest of the oligosaccharide chain from degradation (Suzuki, 1976).

The concentration of sialic acid residues at the surface of red cells and other circulating cells is responsible for the net negative charge of the cells and the mutual repulsive force that prevents aggregation (Pollack, 1965; Seaman, 1975). It is postulated that sialic acids may play a part in keeping various other types of cell separate and that Ca^{2+} and other cations may have a role in forming bridges and allowing cells to come together. It may be that loss of contact inhibition in transformed cell lines is similarly mediated by alterations in the net negative charge of the cells (Schauer, 1982). The trapping of cations by sialoglycoconjugates in cell membranes is presumed to influence ion transport through the membrane although the details are unclear. The specific role of gangliosides in nerve and brain tissue has been much studied; it is postulated that they are involved in ion fluxes associated with action potentials (Brunngraber, 1979, chap. 3).

In general, sialic acids have not been found to contribute to antigenicity despite their prominence on the surfaces of cells and molecules. Horowitz (1978) has examined the hypothesis that sialic acids are so widely distributed that they are "immunological self" and thus non-antigenic. It has been suggested that a factor in the production of neonatal meningitis by group-B meningococci and Escherichia coli K1 is immunological tolerance because of the similarity of their sialic-acid-containing capsules to polysialyl glycopeptides in normal brain (Finne, Leinonen & Makela, 1983). The major sialoglycoprotein of the red cell surface, glycophorin A, has MN blood-group antigen specificity; however the contribution of sialic acid to the antigenic determinant now appears to be indirect

(see below). It is possible, however, to raise antisera against sialic acid haptens in experimental animals; these distinguish between the (2→3) and (2→6) linkages (Smith & Ginsburg, 1980). N-glycolyl sialic acids are not present in man but may be potentially immunogenic and have been implicated in human serum-sickness reactions (Higashi et al., 1977).

In a number of instances sialic acids have been found to mask antigenic determinants formed by other specific groupings on glycoproteins or cell membranes, perhaps by steric hindrance or because of their negative charge. The failure to induce immune responses has been of particular interest in studies of tumour cells, some of which have an increased surface sialic-acid content or altered sialic-acid metabolism (Weiss, 1973; Fishman & Brady, 1976a), and in studies on the role of the trophoblast in preventing immunological rejection of the foetus during pregnancy (Currie & Bagshawe, 1967; Jones & Kemp, 1969). Such studies have produced many conflicting results and the roles of sialic acids in these situations remain unclear (Horowitz, 1978).

The following sections review the evidence for the importance of sialic acids in a number of glycoproteins and cell membranes that have been studied in detail. In most cases the role of the sialic acid is deduced by observing the effect of its removal by neuraminidase action. It may be that this has produced more information on the fate of neuraminidase-treated glycoproteins and cells than on the roles played by sialic acids in normal tissues; however, such studies give an insight into the possible consequences of the production of neuraminidase during infections with pathogenic micro-organisms.

Sialic acid and circulating glycoproteins

Most circulating proteins in mammalian plasma are sialoglycoproteins, containing one or more sialic acid residues per molecule (see Rosenberg & Schengrund, 1976a; Montreuil, 1982). Human plasma sialoglycoproteins have a wide range of biological roles; they include α_1-acid glycoprotein (orosomucoid), alkaline phosphatase, transferrin, ceruloplasmin, immunoglobulins and glycoprotein hormones. It seems probable that the carbohydrate portion of these proteins is involved in giving specificity to their interactions with membranes and cells. The importance of the sialic acid residues has been investigated primarily by treatment with neuraminidase; it should be appreciated that many of the earlier studies used crude enzyme preparations that might give variable hydrolysis of molecules with different linkages, or that might contain other contaminant glycohydrolases that could have produced other additional effects (Gottschalk & Drzeniek, 1972; Wadstrom, 1978).

Human α_1-acid glycoprotein is the major plasma sialoglycoprotein; it has 14 terminal sialic-acid residues on five branched-chain oligosaccharides that are attached near the N-terminal end of the polypeptide (Jeanloz, 1972; Schmid, 1975). Neuraminidase produces a marked change in the electrophoretic mobility of the protein but since the function of this protein remains obscure the effect on its biological activity is unknown. The sialic acid is not part of an antigenic determinant; partial removal of the sialic acids increases its antigenicity (Athineos, Thornton & Winzler, 1962).

In general, neuraminidase treatment of other sialoglycoproteins has similar effects; their electrophoretic mobility is altered but this does not seem to interfere with their specific immunological or biological properties *in vitro* (Winzler & Bocci, 1972; Warren *et al.*, 1978). Thus, enzymic functions of human alkaline phosphatase, amylase, atropinase and cholinesterase are unaffected by neuraminidase, as are the metal-binding properties of ceruloplasmin and transferrin and antigen binding by immunoglobulins (Kristjansson & Cipera, 1963; Johnson *et al.*, 1970; Rosenberg & Schengrund, 1976a). Different degrees of sialylation may be responsible for the microheterogeneity in purified preparations of some of these glycoproteins, e.g. isoenzymes of alkaline phosphatase and amylase.

The glycoprotein hormones are a group of structurally related sialoglycoproteins - luteinising hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) and human chorionic gonadotrophin (HCG). Despite their diverse target cells and physiological actions each consists of two subunits (α and β) of which the α-chain is common to all four hormones. The β-chains are different and confer the specificity of adsorption to receptors on the different target cells. The α-chains are interchangeable *in vitro*; this portion acts to activate adenylate cyclase in the target cell and switch on the appropriate cell function (see Pierce & Parsons, 1981; Cooke, 1982; Stadel, De Lean & Lefkowitz, 1982). Both polypeptide subunits have sialic-acid-containing oligosaccharide chains attached. Many reports have shown that neuraminidase treatment of these hormones does not alter their immunological reactions but does produce a major reduction in their

biological activity *in vivo*, e.g. with FSH (Gottschalk, Whitten & Graham, 1960), LH (Braunstein *et al.*, 1971) and HCG (Barr & Collee, 1967; Van Hall *et al.*, 1971). Similar observations were made with erythropoietin, another sialic-acid-containing glycoprotein hormone (Lowy, Keighley & Borsook, 1960). These findings were initially taken to indicate that sialic acid residues were involved in their specific activities but further investigations have shown that the loss of activity *in vivo* is not paralleled *in vitro* and it is now clear that the primary effect of neuraminidase treatment is to reduce the survival time of the hormones in the circulation.

It seems probable that sialic acids play a general role in determining the fate of a wide range of glycoproteins in mammalian serum. The initial observations by Morell *et al.* (1968) were made with radioactive-labelled desialylated ceruloplasmin. The normal survival time of native ceruloplasmin in the plasma (2-3 days) was dramatically reduced after removal of two or more of the 12 sialic acid residues; it was cleared within a few minutes and was found to have appeared in the cytoplasm of the hepatocytes in experimental animals. It was shown that this depended specifically on the exposure of the subterminal galactose residues after removal of the terminal sialic acids; removal of the galactose residues by galactosidase or modification by treatment with galactose oxidase abolished the effect. Later studies demonstrated that replacement of sialic acid by the action of sialyl-transferase preparations restored the normal survival time of the molecules (Hickman *et al.*, 1970). The recognition of the desialylated ceruloplasmin is dependent on the presence of specific galactose-binding receptors

on the surface of mammalian hepatocytes, and the same mechanism has been shown to produce the accelerated clearance of the glycoprotein hormones and of a wide variety of other desialylated glycoproteins (see Ashwell & Morell, 1974; Hughes, 1976, chap. 13; Ashwell & Harford, 1982; Harford & Ashwell, 1982). After binding of asialyoglycoprotein to the surface receptor the complex is rapidly internalised by endocytosis (receptor-mediated endocytosis); the glycoprotein is catabolised in the lysosomes (Patel, 1978). Only some 10% of the hepatic receptor is present on the cell surface; the role of the remaining 90% inside the cell is still conjectural. The receptor molecules themselves are conserved and recycled in the cell; some of the internal receptor is present in lysosome membranes, apparently on the cytoplasmic surface, but much is also present in Golgi and microsomal membrane fractions (Harford & Ashwell, 1982). Antibody prepared against the hepatic receptor specifically inhibits uptake of desialylated glycoproteins by the liver, which demonstrates that the surface receptor molecules are essential for this process whatever the function of the internal molecules may be (Stockert et al., 1980).

The hepatic receptor has been purified and characterised; it is itself a sialoglycoprotein and the presence of the sialic acid is essential for its ability to bind the exposed galactose residues. There might appear to be similarities between the properties of this receptor and of sialyl- or other glycosyl-transferases, but the isolated receptor cannot be shown to have such enzymatic activity (Hudgin & Ashwell, 1974). The hepatic asialoglycoprotein receptor has many of the properties associated with plant lectins, e.g. binding to a specific sugar moiety,

ability to agglutinate red cells with this sugar exposed on the surface (Stockert, Morell & Scheinberg, 1974) and mitogenic stimulation of desialylated lymphocytes (Novogrodsky & Ashwell, 1977); many authorities now broaden the definition of lectin to include this and similar receptor molecules on the surfaces of mammalian and bacterial cells with specific affinities for particular mono- or di-saccharide residues (Goldstein & Hayes, 1978; Weir, 1980; Barondes, 1981).

The role of the mammalian hepatocyte receptor in removing injected asialoglycoproteins is clear; the role of this mechanism in determining the survival time of native sialoglycoproteins in normal animals is less certain. It is attractive to postulate that ageing molecules of circulating glycoproteins lose their sialic acid residues and are preferentially removed from the circulation; indeed the turnover of sialoglycoproteins might be partially regulated by the rate of removal of sialic acid, whether enzymically or by chemical forces. The site of this postulated desialylation in normal mammals is unknown however, though some neuraminidase activity may be present in normal plasma (Warren & Spearing, 1960) and in the surface membranes of hepatocytes and other cells (Rosenberg & Schengrund, 1976b). Furthermore, this mechanism for glycoprotein scavenging is restricted to mammals, which argues against a general role for sialic acid in such a process. Birds do not have a galactose-specific hepatic receptor and, in contrast to the situation in mammals, a high proportion of circulating glycoproteins are already partially desialylated. Further degradation to expose N-acetyl glucosamine leads to rapid

clearance from the plasma by an analogous glucosamine receptor in chicken hepatocytes (Lunney & Ashwell, 1976). Equivalent mechanisms have not yet been identified in other phylogenetic groups.

Despite some areas of uncertainty it seems reasonable at present to assume that the hepatic receptor does have a physiological role and that it is likely to play a part in determining the survival time of circulating glycoproteins. Some glycoproteins do not contain sialic acid but do contain galactose; it may be that this is normally masked, e.g. by terminal fucose residues, and that such proteins are also removed when the galactose residues are exposed (Clamp, 1975). It has been shown that mammalian liver damage may be associated with detectable amounts of circulating desialylated glycoproteins (Harford & Ashwell, 1982). Evidence that an analogous system is involved in determining the lifespan of circulating erythrocytes is discussed below.

Erythrocyte sialic acid and blood group antigens

A variety of lines of investigation followed from the recognition of sialic acid as the essential constituent of the erythrocyte virus receptor in the early studies on interactions of influenza viruses and red blood cells (see Gottschalk, 1960). Interest in the broader role of the membrane-associated sialic acid was stimulated when it was shown that the negative charge of the erythrocyte is attributable to its sialic acid content (Eylar *et al.*, 1962). Neuraminidase treatment reduces the electrophoretic mobility of the red cells, rendering them more easily aggregated or

agglutinated by antisera under a variety of physico-chemical conditions (Pollack, 1965). It became apparent that a substantial part of the erythrocyte sialic acid was present in glycoprotein rather than glycolipid when Cook, Heard & Seaman (1960) demonstrated that treatment with proteolytic enzymes releases sialoglycopeptides from the red cell surface. Early studies on the human ABOH blood-group substances suggested that they were predominantly present in glycolipids in the red cell membrane. Neuraminidase treatment did not affect ABO specificity but did destroy the MN blood-group antigens (Klenk & Uhlenbruck, 1960; Cook & Eylar, 1965). Satisfactory techniques for extraction and analysis of membrane components proved difficult to develop until the 1970s when a variety of methods were used to produce sialoglycoprotein extracts with MN activity (Hamaguchi & Cleve, 1972; Anstee & Tanner, 1974). Red-cell surface structures have since been intensively investigated, partly because of the intrinsic interest of this cell and its clinical importance in blood transfusion, and partly because of its appeal as a simple model cell for studies on general problems of mammalian cell-membrane structure. Although sialic acid has been implicated in many different processes involving the red cell surface, it remains difficult to determine its precise biological role.

The immunochemistry of human red-cell antigens was intensively studied in the 1950s and 1960s with particular reference to the ABOH, P, I and Lewis family of related antigenic specificities (see Morgan & Watkins, 1969; Watkins, 1972; Marsh, 1981; Mollison, 1983, chap. 7). These antigens are present in

cell-surface glycoconjugates that contain fucose as a terminal sugar but not sialic acid; however, a brief account is given here because of the influence of these findings on later studies of the MN system. The initial investigations did not use antigen extracted from red cells, but glycoproteins in various secretions that were found also to have ABH blood-group activity. The antigenic specificities depend on the sequence and linkage of sugars in the oligosaccharide chains rather than on differences in the protein part of the molecule; the antigens are synthesised by sequential addition of sugars to the precursor substance. The gene products are glycosyl-transferase enzymes, and the specific antigenic oligosaccharide results from the particular complement of glycosyl-transferases specified in the individual's genotype (see table Intro/II). The enzymes may act on a variety of precursors in the cell membrane, or in plasma and secretions, producing the same antigenic specificity. More recently techniques have been evolved for studying the erythrocyte membrane components directly (Marchesi, 1979). Such studies have confirmed the picture that emerged from the work on blood-group-active glycoproteins in secretions and have shown that the antigenic oligosaccharides are attached to several different components in the red cell membrane, including simple glycolipids, polyglycosylceramides and glycoproteins (see Hakomori, 1981).

The current concept of red-cell membrane structure was discussed by Lux (1979) and by Mollison (1983, chap. 6). The general fluid-mosaic model of Singer & Nicolson (1972) postulated a fluid lipid bilayer with various proteins embedded in it, forming a dynamic mosaic on the surface of the membrane. Marchesi (1979)

TABLE Intro/II

Structure of terminal oligosaccharides determining human blood-group ABH antigenic specificity

Blood-group substance	Gene product	Terminal structure of oligosaccharide[a]
Precursor	-	Gal (β1-3) GlcNAc → R
H	α-L-fucosyl transferase	Gal (β1-3) GlcNAc → R; Fuc (α1-2) Gal
A	α-N-acetyl-D-galactosaminyl transferase	GalNAc (α1-3) Gal (β1-3) GlcNAc → R; Fuc (α1-2) Gal
B	α-D-galactosyl transferase	Gal (α1-3) Gal (β1-3) GlcNAc → R; Fuc (α1-2) Gal

[a] From Mollison (1983).

proposed a modification of this for the erythrocyte, with a fixed protein matrix beneath the lipid bilayer. The major structural protein of the red cell, spectrin, occurs on the inner surface of the membrane only; it is thought to interact with actin and other internal proteins to form a supporting layer. Some glycoproteins at least are thought to be relatively fixed in the red cell membrane; these are postulated to be transmembrane proteins with a tail that penetrates the lipid bilayer and may interact with the structural matrix below.

Hughes (1976, chap. 4) reviewed the evidence that integral membrane glycoproteins are generally amphipathic, with a hydrophobic portion inserted into the lipid membrane and a hydrophilic portion, bearing the oligosaccharide chains, that extends into the aqueous environment. Perhaps the best studied membrane glycoproteins are those of the human red cell. The proteins can be separated and identified by the SDS-PAGE technique (Marchesi, 1979). Aggregates of one major transmembrane glycoprotein, the so-called band-3 protein, form the anion channel of the red cell membrane; this protein does not contain sialic acid and its external carbohydrate portion carries the ABH and I blood-group determinants. At least four sialoglycoproteins have been identified, of which two have been purified and characterised - glycophorin A (α-sialoglycoprotein) and glycophorin B (δ-sialoglycoprotein); various homo- and hetero-dimers and larger complexes are also observed (Anstee, 1981). These glycoproteins account for the majority of the total red-cell sialic acid, although some is also present in glycolipids. Glycophorin A (MW 31,000) is the major sialoglycoprotein component of the red

cell surface and carries the MN blood-group specificity; there are c. 10^6 molecules of glycophorin A on each red cell. Glycophorin B also has N-group activity and in addition carries the associated Ss specificity.

The observation that MN blood-group activity was abolished by neuraminidase was initially interpreted to suggest that sialic acid formed part of the antigenic determinant. By analogy with the ABH system it was argued that the MN gene products were likely to be glycosyl-transferases. M and N are allelic genes but some N antigen is often detectable in M cells; this led to the suggestion that the N substance was a monosialyl oligosaccharide that was the precursor for M, with the M-gene product a second sialyl-transferase that was required for complete sialylation of the glycoprotein (Springer, Tegtmeyer & Huprikar, 1972; Watkins, 1974). Other workers, however, were unable to confirm consistent differences in the oligosaccharides isolated from M and N cells (Blumenfeld & Adamany, 1978); the structure of the carbohydrate side chains of normal glycophorin A is shown in table Intro/III (T and Tn antigens are discussed below).

More recent studies have disproved this theory and it is now clear that the primary difference between glycophorin A from M and N cells lies in the polypeptide part of the molecule (Walker, Rubinstein & Allen, 1977; Sadler, Paulson & Hill, 1979). The complete amino acid sequence of the protein was reported by Tomita, Furthmayr & Marchesi (1978). The polypeptide chain contains 131 amino acids and spans the membrane with its amino terminus outside the cell and its carboxy terminus inside; about 70 amino acids are external to the membrane, 20 within it, and 40 in the cytoplasm.

TABLE Intro/III

Structure of O-glycosidically linked oligosaccharides of glycophorin A in normal, T and Tn red blood cells

Red cell antigen	Oligosaccharide structure[a]
Normal (M/N)	NANA (α2-3) $\longrightarrow$ Gal (β1-3) $\longrightarrow$ GalNAc $\longrightarrow$ Ser/Thr; NANA (α2-6) $\uparrow$ GalNAc
T	Gal (β1-3) $\longrightarrow$ GalNAc $\longrightarrow$ Ser/Thr
Tn	GalNAc $\longrightarrow$ Ser/Thr

[a] From Anstee (1981).

The M and N genes specify polypeptide chains that differ only at positions 1 and 5, as shown in table Intro/IV. Glycophorin A has a high carbohydrate content (c. 60%); it contains 15 O-glycosidically linked oligosaccharides, of the structure shown in table Intro/III, and one N-glycosidically linked oligosaccharide, of different though still debated structure. The carbohydrate chains are all attached to the portion of the molecule external to the membrane; three of the O-glycosidically linked oligosaccharides are attached to amino acids in positions 2, 3 and 4. The MN antigenic activity is conserved in octaglycopeptides containing only the terminal eight amino acids and these three oligosaccharide chains (Dahr & Uhlenbruck, 1978). Glycophorin B has a different polypeptide chain; the terminal octapeptide is invariable but the same as that giving N specificity in glycophorin A (Anstee, 1981).

Although sialic acid is essential for M and N antigenic activity it does not form part of the antigenic determinant. Chemical modification either of the sialic acids (Ebert, Metz & Roelcke, 1972) or of the free amino groups (Lisowska & Duk, 1975) interferes with MN activity; both components are necessary for the specific antigenic conformation. Neuraminidase abolishes both M and N activity; resialylation of glycophorin A with specific sialyl-transferases restores the original M or N specificity (Sadler et al., 1979). Lectins with anti-N activity bind to the carbohydrate portion of glycophorin A, but to internal sugars and not to the sialic acids (Dahr & Uhlenbruck, 1971; Anstee, 1981). It appears that the primary differences in the amino acids at positions 1 and 5 determine the spatial configurations of the

TABLE Intro/IV

Amino acid sequences of the amino-terminal octaglycopeptide of glycophorin A from human red cells of blood groups M and N

Red cell antigen	Structure of terminal octaglycopeptide[a]
M	Ser - Ser(O-glycosidically linked oligosaccharide) - Thr(O-glycosidically linked oligosaccharide) - Thr(O-glycosidically linked oligosaccharide) - Gly - Val - Ala - Met -
N	Leu - Ser(O-glycosidically linked oligosaccharide) - Thr(O-glycosidically linked oligosaccharide) - Thr(O-glycosidically linked oligosaccharide) - Glu - Val - Ala - Met -

[a] Modified from Anstee (1981); ♀ = O-glycosidically linked oligosaccharide (see table Intro/III).

oligosaccharide chains so that different antigenic conformations of the common internal sugars are exposed; this is thought to depend on the charge interactions between the amino acids and the carboxyl groups of the sialic acids (Furthmayr, 1978; Anstee, 1981). Thus the frequently repeated statement that sialic acid plays a role in MN blood-group specificity is true, albeit in a less direct way than was originally envisaged.

T-polyagglutinability and red cell lifespan

The clarification of the role of sialic acid in MN blood-group specificity has also allowed a clearer understanding of the immunochemistry of the Thomsen-Friedenreich phenomenon. Neuraminidase treatment of erythrocytes not only destroys MN antigens but also exposes the cryptantigen T on the cell surface. It was originally assumed that T would prove to be the precursor for the M or N antigens, but it is now evident that this was an over-simplification. Bird (1964) described a lectin from peanuts (Arachis hypogaea) that has anti-T activity and this has been widely used in studies of T-polyagglutinability. It binds the disaccharide galactose ($\beta 1 \rightarrow 3$) N-acetyl galactosamine; this is the structure exposed by removal of the two molecules of NANA from the oligosaccharide of glycophorin A (see table Intro/III). Only a small proportion of the cell-surface sialic acid is involved in the MN antigenic determinants - that in the O-glycosidically linked oligosaccharides attached close to the amino terminus of glycophorin A (and probably also of glycophorin B). There are many more potential T-antigen sites on the red cell surface, as T is

also exposed by removal of sialic acid from any of the other O-glycosidically linked oligosaccharides attached to other parts of the glycophorin molecules or to glycolipids in the cell membrane (Anstee, 1981).

Panagglutination was the term originally used to describe the agglutination of red cells by naturally occurring antibodies independent of the ABO system that are present in a wide range of normal vertebrate sera. The cells are now more usually described as polyagglutinable rather than panagglutinable because the antibodies concerned may be common rather than universal. A variety of mechanisms other than the T/anti-T system may be responsible for polyagglutinability, e.g. adsorption of bacterial antigens to the surface of red cells, or exposure of other crypt-antigens by the action of other bacterial enzymes, or the presence of very rare genetic blood groups to which natural antibodies commonly occur (see Prokop & Uhlenbruck, 1969; Mollison, 1983, chap. 10). Table Intro/III also shows the structure of the Tn antigen, which gives rise to a different type of polyagglutinability; in this case the chain terminates with the N-acetyl galactosamine residue. Anti-Tn occurs naturally in the serum of many normal people; a specific anti-Tn lectin from Salvia sclarea is also available (see Bird, 1981). Tn cells do not arise by enzymic removal of the distal sugars as do T cells, but rather by a somatic mutation that gives rise to a clone of cells that lack the glycosyl-transferases necessary for elaboration of the oligosaccharide (Anstee, 1981).

Anti-T occurs in variable (often low) titres in the serum of man and other animal species; it is usually IgM in nature, and

agglutination of neuraminidase-treated cells is best demonstrated at room temperature or in the cold (Mollison, 1983, chap. 10). Its origin is debated; presumably it might arise by exposure of T antigen during synthesis or catabolism of red-cell surface structures. In general, it is not found in umbilical cord blood and this argues for external factors in stimulating antibody production. Springer & Tegtmeyer (1981) have demonstrated the production of anti-T by chicks in response to administration of particular strains of commensal Escherichia coli with fortuitous T-antigen activity; the same mechanism is invoked to explain the presence of natural anti-A and anti-B associated with the ABO blood-group system. The presence of natural anti-T in sera used for testing for other blood-group antigens may give rise to misleading or conflicting results; however, the anti-T is usually diluted to insignificant proportions when high-titre immune diagnostic antisera are used. High-titre IgG anti-T may be produced in rabbits in order to give a reliable diagnostic reagent for investigation of polyagglutinability; increasingly the peanut lectin and a variety of other lectins are used for detection of T and a number of related variant antigens (see Bird, 1981).

T-polyagglutinability is essentially an in-vitro phenomenon, where red cells can be shown to have been altered by enzyme treatment so that anti-T serum or lectins will produce agglutination. It may also occur in vivo, with red cells from a patient found to be polyagglutinable during routine cross-matching. On occasion this has occurred in patients shown to have infections with myxoviruses or bacteria that can produce polyagglutinability in vitro; only a small proportion of such cases have shown

haemolysis or other effects that might be attributable to interaction of altered red cells with natural anti-T (see Chorpenning & Hayes, 1959; Bird & Stephenson, 1973; Klein et al., 1977; Seger et al., 1980; Bird, 1981). Unfortunately, several of the reported cases have not been well documented and, since the polyagglutinability has been assumed to involve T exposure without adequate investigation, other mechanisms of polyagglutinability and of haemolysis might have been involved; in particular, haemolysis during severe Clostridium perfringens infections might also be attributed to phospholipase-C (α-toxin) action.

The significance of possible exposure of T antigen and its combination with natural anti-T in vivo remains unclear (see Mollison, 1983, chap. 10). T-activated cells fail to agglutinate in their own serum. It is possible that the patient's anti-T is absorbed by his own cells, though the cells give a negative direct antiglobulin reaction. Anti-T is often a cold-reactive agglutinin, apparently ineffective at 37°C. Other red cell autoantibodies are known that react predominantly in the cold and that do not appear to cause damage in vivo, e.g. anti-I cold agglutinins in normal sera; there are also a few instances of harmless warm autoagglutinins. Tn cells (see above) are genetic variants with an exposed cryptantigen related to the T antigen. Anti-Tn is present in many normal people though not in patients who are producing these cells; they do not appear to have a reduced survival time in plasma unless transfused into a person with circulating anti-Tn (Myllyla et al., 1971). There are conflicting reports about the effect of infusion of blood containing natural

anti-T into patients whose cells have T antigen exposed; only in a few cases has haemolysis been reported (Seges et al., 1981).

Red cells treated with neuraminidase in vitro have been shown to be rapidly eliminated when reinjected. It should be borne in mind that early experiments used crude enzyme preparations that might remove variable amounts of sialic acid and that might contain other enzymes that could produce other effects at the red cell surface (Wadstrom, 1978). Similarly it should be appreciated that other changes may be produced, e.g. neuraminidase or other bacterial antigens may be adsorbed to the cell surface during such treatment (Sedlacek & Seiler, 1974; Schauer, 1982). The mechanism of elimination of neuraminidase-treated red cells, and the relative significance of cryptantigens, antibody or altered surface charge in this process have been debated (see Jancik et al., 1978).

Lee (1968) showed that neuraminidase-treated red cells were more susceptible to phagocytosis by peritoneal macrophages; serum factors were required for opsonisation. Kay (1975) demonstrated a requirement for antibody for phagocytosis of treated red cells. Since IgG antibody could be demonstrated adsorbed to the surface of old cells but not young cells in normal blood, she argued that this was the normal mechanism for elimination of ageing red cells. Durocher, Payne & Conrad (1975) found that after neuraminidase treatment red cells were sequestered in the liver and suggested that they might be recognised by a mechanism similar to that described for desialylated glycoproteins.

Although glycoproteins may be removed by hepatocytes, red cells do not come in contact with hepatocytes and would be more likely to be removed by Kupffer cells. Aminoff et al. (1977) found

that removal of 10% of the surface sialic acid produced rapid sequestration of red cells in the liver and the spleen. There was specific adhesion of treated red cells to liver Kupffer cells and to spleen macrophages though not to hepatocytes; they noted a number of differences between the Kupffer-cell receptors and the classic hepatocyte receptor. These results contrast with those of Kolb et al. (1978b), who demonstrated that both Kupffer cells and hepatocytes have galactose-specific receptors; further analysis of the receptors by inhibition studies failed to show any difference in specificity between the receptors on the two types of cell (Kolb et al., 1980). The uptake of neuraminidase-treated red cells by the liver can be inhibited in vivo by injection of substances with exposed galactose residues (Kolb, Friedrich & Suss, 1981; Muller, Franco & Schauer, 1981); this confirms the importance of the galactose receptor in vivo and argues against the involvement of antibody in this process. Kuster & Schauer (1981) demonstrated that the initial binding of treated red cells to Kupffer cells occurs in buffer but that serum components, probably complement, are required for phagocytosis thereafter.

Thus, although there remain a number of inconsistencies and uncertainties, there is reasonable evidence to suggest that neuraminidase-treated red cells are removed by phagocytic cells in the liver and spleen following recognition of galactose residues exposed on the red cell surface. The question arises whether this mechanism might also be responsible for removing ageing red cells in the normal situation (see Schauer, 1982). The red cell has a lifespan of c. 120 days, during which it travels some 300 miles. Every day c. 2×10^{11} cells (c. 1% of the total) are removed from

the circulation, primarily in the spleen but also by the liver (which can take over completely after splenectomy). There is a little random destruction but in general the oldest cells are removed preferentially. One suggested mechanism is that as these non-nucleated cells age various biochemical processes begin to "wear out" and the cells become more rigid. This loss of deformability is postulated to lead to more damage while passing through the capillaries and eventual trapping in the spleen; the reduced lifespan in some hereditary red-cell abnormalities lends support to this hypothesis (Berlin & Berk, 1975; Chien, 1975; Lux, 1979).

An alternative hypothesis suggests that ageing red cells lose sialic acid and that the liver and spleen provide a quality-control mechanism so that after a critical amount has been lost the cells are removed from the circulation. Such a mechanism might act non-specifically, i.e. the loss of sialic acid in itself alters membrane charge or other properties so that the cells can be recognised, or it might require specific exposure of the sub-terminal galactose residues for interaction with the specific phagocyte receptors in spleen or liver. It has proved difficult to demonstrate that there is specific loss of sialic acid from ageing red cells. Erythrocytes can be fractionated on the basis of their density (Berlin & Berk, 1975); the oldest cells are the most dense and studies have been made to determine their relative surface-charge density and sialic acid content (see Schauer, 1982). Although such cells do contain 10-20% less sialic acid it appears that they have also lost other membrane components in the same proportions (Seaman et al., 1977) and there is no evidence for

reduced sialylation of glycophorin A in older cells (Lutz & Fehr, 1979). Thus sialic acid loss may be by loss of whole membrane fragments rather than by selective hydrolysis to expose the subterminal galactose residues, and it appears unlikely that ageing red cells resemble neuraminidase-treated cells in this way.

Discussions of the non-specific role of sialic acid at the red cell surface focus on the importance of the net negative surface charge of these circulating cells. Many studies have demonstrated increased agglutinability by "incomplete" antibodies after removal of sialic acid by neuraminidase or protease treatment; the agglutinability of the red cell correlates with its sialic acid content, its electrophoretic mobility and the effective zeta potential under the test conditions (see Pollack, 1965; Seaman, 1975). It is less clear, however, whether the surface sialic acid is important in preventing aggregation or phagocytosis in vivo. One line of argument against an important role for sialic acid in the normal red cell is the existence of a number of rare red cell types that are relatively deficient in sialic acid. The normal lifespan of Tn cells was mentioned above. A very rare type of red cell, called En(a-), has been shown to be associated with complete absence of glycophorin A; there is a little compensatory increase in sialylation of other red-cell surface components but the cells have only 40% of the normal NANA content (Tanner & Anstee, 1976). The even rarer homozygous M^k cells lack both glycophorins A and B and have only 25% of the normal NANA. These cells are first recognised during cross-matching or because of transfusion reactions but they may be found with apparently normal lifespan in perfectly healthy people in the few families in which

they have been recognised (Anstee, 1981). These observations, though involving a very few people with very rare genetic abnormalities, make it difficult to postulate an important general role for sialic acid in the life of the erythrocyte.

Although we do not yet have an adequate picture of the role of sialic acids in the normal economy of the red cell, the evidence discussed above suggests that removal of sialic acid by neuraminidase during the course of an infection might have adverse effects on the patient's red cells, producing antigenic changes, adsorption of antibody, haemolysis or enhanced clearance from the circulation. The following section discusses other systems that might be affected by exogenous neuraminidase.

Other roles for sialic acids

The roles of sialic acids in circulating glycoproteins and on the erythrocyte membrane have been intensively investigated as reviewed above; studies on a number of other biological systems and cell types have also suggested that sialic acids may play important roles although, in general, the evidence is still incomplete.

Reutter *et al.* (1982) have reviewed the involvement of sialic acid in blood clotting. Neuraminidase treatment of thrombocytes removes sialic acid, exposes T antigen and leads to rapid removal from the cirulation as with erythrocytes (Choi, Simone & Journey, 1972). Membrane-bound sialic acid is thought to play a role in adhesion and aggregation of thrombocytes. Several of the plasma factors involved in blood clotting, e.g. plasminogen, fibrinogen

and Von Willebrand factor, are sialylated and there is evidence that removal of the sialic acid groups affects their functions.

Lymphocytes also have sialic acid on their surface and it has been shown to be important in the distribution and processing of these cells in the body. Neuraminidase-treated lymphocytes are selectively trapped in the liver rather than in the spleen or lymph nodes (Woodruff & Gesner, 1969). Unlike neuraminidase-treated erythrocytes, they are not destroyed there; after c. 24 h their surface sialic acid has been regenerated and they re-emerge to resume the normal pattern of recirculation (Woodruff & Woodruff, 1974, 1976a and b). Kolb *et al.* (1978a) showed that the trapping of lymphocytes was effected by the hepatocyte galactose-specific asialoglycoprotein receptor. Rose (1982) discussed the possibility that immature lymphocytes have exposed T antigen that binds peanut agglutinin and suggested that the lectin-binding properties of lymphocytes may reflect their tendency to recirculate or be sessile. Sialic acid may not play a part in the normal pattern of mature lymphocyte recirculation and processing but it is clearly important in preventing temporary trapping in the liver. Novogrodsky & Ashwell (1977) and Kolb-Bachofen & Kolb (1979) have discussed the importance of this effect *in vivo* during myxovirus infections and the possible induction of autoimmune cytotoxic reactions in the liver following mitogenic stimulation of trapped lymphocytes by contact with the hepatic receptor (lectin).

Sialic acids are prominent surface structures on many other cells in the body besides the circulating blood cells described above and it is thought that they play a role in a wide range of

surface-dependent phenomena, e.g. adhesiveness or mutual repulsion of like cells, cell differentiation, or interactions between different cell types (see Reutter et al., 1982). Neuraminidase treatment of many types of cell leads to enhanced cell destruction, often mediated by serum factors; Ray (1977) discussed the role of such mechanisms in immune surveillance for clearing defective, aged, mutated or malignant cells. Variations in the amount of sialic acid on the surface of transformed cells and tumour cells have been extensively investigated but no general role can confidently be ascribed to sialic acid in these phenomena (Jeanloz & Codington, 1976; Ray, 1977; Glick & Flowers, 1978; Horowitz, 1978; Reutter et al., 1982). Sialic acid is postulated to have a masking effect at the cell surface, either directly, when removal of sialic acid creates a new antigen involving the subterminal sugar as with T antigen, or indirectly, when the bulky, charged sialic-acid residue is presumed to block access to less superficial groupings (Jeanloz & Codington, 1976). It appears that certain tumours with reduced surface sialic acid express T antigen or other cryptantigens although this fails to provoke an effective immune response; immunotherapy with neuraminidase-treated cells may lead to tumour regression or reduce metastasis (see Ray, 1977; Bird, 1981; Springer & Tegtmeyer, 1981; Reutter et al., 1982).

In general, sialic acid has not been found to be part of antibody receptors on tissue cells although the charge effects of these molecules might affect the antigenic conformation of other cell-surface components indirectly as with erythrocyte MN antigens. The major histocompatibility antigens on lymphocytes and other tissue cells are sialoglycoproteins but the antibody receptor

appears to lie in the protein part of the molecule (Hughes, 1976, chap. 6; Horowitz, 1978). On the other hand, sialic acid is known to be part of cell-surface receptors for a variety of other agents (see Jeanloz & Codington, 1976; Schauer, 1982). A number of these examples have been studied primarily with erythrocytes but similar surface structures occur widely in membrane glycoproteins and gangliosides of other cells.

The importance of sialic acid in myxovirus receptors is well established. The detailed structure of glycophorin A, the major virus receptor on the erythrocyte surface, was discussed above, and similar NANA-containing glycoconjugates act as virus receptors on other types of cell. The inter-related reactions of viral haemagglutinin and neuraminidase with the virus receptors are discussed below. Polyoma viruses also bind to sialic acid receptors on red cells; this is not merely attributable to charge interactions as there is specificity for (2 → 3) linked but not (2 → 6) linked molecules (Cahan & Paulson, 1980). A similar receptor has also been described for a number of mycoplasmas, e.g. Mycoplasma pneumoniae, which adsorb to erythrocytes and other cells using receptors that contain sialic acid (Sobeslavsky, Prescott & Chanock, 1968; Glasgow & Hill, 1980).

Two lectins, from the horseshoe crab (Limulus polyphemus) and from wheat germ (Triticum vulgaris), attach to receptors with terminal sialic-acid residues (see Jeanloz & Codington, 1976; Schauer, 1982). Although these lectins do not normally come in contact with human cells, it is assumed that lectins frequently bind to physiologically important receptors on the cell surface as they may stimulate a variety of important cell reactions (Barondes,

1981); the lymphocyte mitogenic response to purified hepatocyte galactose receptor (lectin) was cited above.

Jeanloz & Codington (1976) evaluated the evidence for sialic acid residues in the receptor sites for a number of hormones, including insulin, adrenocorticotrophic hormone and serotonin (see also Van Heyningen, 1974; Fishman & Brady, 1976b). The importance of sialic acid in the hepatocyte galactose receptor was mentioned previously. Sialic acid has also been found to be important in ganglioside receptors for interferon (Besancon & Ankel, 1974). In all these cases, neuraminidase treatment interferes either with binding or with later stages in the transfer of information across the cell membrane.

Gangliosides have been identified as receptors for a number of bacterial toxins (see Reutter et al., 1982). Van Heyningen (1959) demonstrated the binding of tetanus toxin to gangliosides extracted from nervous tissue and showed the importance of the NANA residues in this interaction (Bernheimer & Van Heyningen, 1961). The toxin is bound to the monosialoganglioside GM1 (see table Intro/I); binding is lost in the absence of the NANA residue and very much increased with di- and tri-sialogangliosides (Van Heyningen, 1974). Brunngraber (1979, chap. 3) summarised the current concepts of tetanus-toxin-binding to peripheral nerve, intra-axonal retrograde transport to the spinal cord, and action at the motor synapse. It is probable that the ganglioside receptor is involved only in the initial transport of the toxin into the nerve cell rather than in its final action in the central nervous system (Stephen & Pietrowski, 1981, chap. 2).

Cholera toxin also binds to a ganglioside receptor at the cell surface (see Finkelstein, 1976; Fishman & Brady, 1976b; Van Heyningen, 1977). Cholera toxin consists of two types of subunit; the B subunits are responsible for binding to the cell surface while the A subunit is responsible for activation of adenylate cyclase and the toxic effects on the target cell. The toxin binds specifically to ganglioside GM1 and is neutralised by it; di- and tri-sialogangliosides are ineffective (Van Heyningen, 1974). The susceptibility of various cells to cholera toxin is proportional to the amount of GM1 that is naturally present or that can be introduced into their surface membrane (Cuatrecasas, 1973; Hollenberg et al., 1974); the number of available receptors can be increased by neuraminidase treatment, which removes NANA from various tri- and di-sialogangliosides to produce GM1 (Haksar, Maudsley & Peron, 1974). Moss, Manganiello & Fishman (1977) produced evidence that both the terminal galactose and the "internal" sialic acid are important in binding of cholera toxin B subunit by GM1. The mechanism of activation of adenylate cyclase by subunit A is still not completely understood, but it seems that the A subunit has to be transported to the inner surface of the cell membrane where it has an enzymic action (ADP-ribosyl transferase) that inactivates a component of the adenylate cyclase complex; this prevents GTPase activity and traps the adenylate cyclase in the active form (see Holmgren, 1981). The role of the ganglioside in the initial binding of the B subunit is well established; it is not known what part it plays in the subsequent transfer of the A subunit through the membrane.

Thus, sialic acids are known to form an essential part of a number of cell-surface receptors for various agents. Although it is hard to claim a physiological role for some of the systems that have been studied, e.g. with toxins and lectins, it seems probable that these agents are interacting with systems that do have a normal role in information transfer over cell membranes that can lead to changes in the behaviour of the cell. A physiological role is more directly demonstrated with the hepatocyte galactose-specific receptor and receptors for hormones and interferon. Viruses and bacterial toxins may exploit the presence of receptors that have a quite separate role and turn this to their own advantage. In some cases the sialic acid may be involved only in primary binding of the agent but this may initiate a chain of other interactions that alters the behaviour of the cell. The evidence from studies of this sort supports the suggestion that sialic acids are essential constituents in a variety of sialoglycoconjugates that have important regulatory roles at cell surfaces; this in turn suggests that neuraminidase produced during infection by pathogenic micro-organisms might interfere with important functions in the host's tissues.

MAMMALIAN NEURAMINIDASE

Neuraminidases that occur in mammalian tissues have proved very much more difficult to purify and have been very much less well characterised than the enzymes from bacterial or viral sources. The presence of the enzyme in mammals was first reported by Warren & Spearing (1960) who detected activity in concentrated fractions prepared from pooled human plasma, but the levels of free enzyme in plasma and other body fluids, e.g. saliva and milk, are usually very low (Schauer et al., 1976). Neuraminidase activity has since been detected in a wide range of mammalian and avian tissues, where it is mainly associated with lysosomes and other membrane fractions (Carubelli, Trucco & Caputto, 1962; Mahadevan, Nduaguba & Tappel, 1967; Tuppy & Palese, 1968; Patel, 1978). The intracellular distribution of the enzyme has been studied particularly in liver, kidney and brain tissue where the levels are highest, and evidence has been found for the presence of more than one type of enzyme, with different pH optima and substrate specificities. Many conflicting results have been obtained, largely attributable to differences in fractionation procedures, the use of crude preparations and inappropriate assay conditions, but the general picture is becoming a little clearer.

Many of the enzymes studied have the acidic pH optimum (4.0-4.5) characteristic of lysosomal enzymes, while others have a higher pH optimum (5.0-6.0) that may be more appropriate for enzymes active elsewhere in the cell or on the cell surface (see Rosenberg & Schengrund, 1976b). The lysosomal enzymes do not usually show the dependence on calcium and other ion concentrations

that is characteristic of a number of microbial enzymes, though this may vary with the substrate involved; for example, Schengrund & Nelson (1975) found a requirement for added Ca^{2+} with bovine brain neuraminidase acting on exogenous ganglioside substrates but not on endogenous gangliosides. Varied susceptibilities to inhibition by metallic ions, e.g. Hg^{2+} and Cu^{2+}, and to enhancement by detergents have also been reported (Rosenberg & Schengrund, 1976b); these have been useful in distinguishing the presence of different enzymes in various tissues but it is difficult to generalise from the results because of the varying degrees of purification involved.

Activity against different substrates also varies markedly. Mammalian enzymes can be roughly characterised either as glycoprotein enzymes, that give rapid hydrolysis of glycoproteins and oligosaccharides (e.g. sialyl-lactose) but that have little activity against gangliosides, or as glycolipid enzymes that have the converse substrate affinities (Schauer, 1982). When defined substrates are used, variations in relative activity against (2 → 3), (2 → 6) and (2 → 8) linkages have been shown, but it appears that the lysosomal enzymes can commonly hydrolyse all three types (Corfield & Schauer, 1982b). Few enzymes have significant activity against the "internal" NANA in intact gangliosides; this residue is usually protected until the distal part of the oligosaccharide has been removed by sequential action of the appropriate enzymes (Suzuki, 1976; Li & Li, 1982).

The subcellular distribution of neuraminidase activity has been studied in a variety of tissues but the picture remains unclear. Most of the enzyme is membrane-bound and it has been

shown in membrane fractions thought to represent constituents of several different cell structures, e.g. in rat liver it has been found in lysosomes (Horvat & Touster, 1968), Golgi apparatus (Kishore et al., 1975) and plasma membrane (Visser & Emmelot, 1973). Studies on neuraminidase distribution in brain tissue have proved difficult; the enzyme is found mainly in the grey matter and is particularly associated with synaptosomal membrane fractions (Patel, 1978). Synaptosomal neuraminidase is active against endogenous gangliosides present in brain fractions and it is postulated to play a role in synaptic transmission (Brunngraber, 1979, chap. 12). It is unclear whether reports of soluble enzyme activity (e.g. Tulsiani & Carubelli, 1970) indicate a true cytosol enzyme or a membrane-associated enzyme that is easily solubilised during tissue extraction procedures; the pH optimum is usually higher than for lysosomal enzymes. Recently Michalski, Corfield & Schauer (1982) demonstrated two forms of neuraminidase in human liver lysosomes; one was membrane-bound and insoluble but the other could be solubilised in the presence of Triton X-100 and purified. The latter enzyme, one of the best purified mammalian neuraminidases, had MW 70,000, pH optimum 4.0-4.5 and activity primarily against gangliosides; the enzyme remaining firmly bound to the lysosomal membrane fraction was of the glycoprotein type.

Disease due to neuraminidase deficiency

Further insight into the role of human neuraminidase has come with the demonstration that certain rare genetic diseases are associated with defects in neuraminidase production. A large

number of metabolic diseases have been identified and classified by various schemes, initially on clinical grounds by eponyms (Gaucher disease, Tay-Sachs disease, etc.) but more recently by biochemical criteria in evolving and overlapping categories such as glycosphingolipidoses, gangliosidoses, mucolipidoses, oligosaccharidoses or sialidoses (see Tallman & Brady, 1976; Brunngraber, 1979, chap. 14; Brady, 1982). They are characterised by excessive tissue accumulation and/or urinary excretion of gangliosides or glycoconjugate-derived oligosaccharides; in a number of cases they can be attributed to a primary defect in a specific lysosomal hydrolase. Cantz, Gehler & Spranger (1977) first identified a primary defect in neuraminidase production as the cause of a sialidosis when they showed an almost complete lack of the enzyme in fibroblasts cultured from the skin of a patient with mucolipidosis I. Further studies have identified at least two syndromes associated with primary neuraminidase deficiency (Cantz, 1982). One group of patients present in infancy with severe progressive neurodegenerative disease and a dysmorphic syndrome similar to Hurler disease; there may be associated renal damage. Others have a milder form with normal physical appearance and the onset of neurological and ophthalmic signs delayed till adolescence. These syndromes are thought to result from defects in neuraminidase production, probably due to allelic mutations in the same neuraminidase gene giving rise to different degrees of lysosomal neuraminidase deficiency.

The biochemical diagnosis of these conditions depends on analysis of the oligosaccharides stored in the tissues and excreted in the urine, and on demonstration of lysosomal enzyme defects in

cultured fibroblasts or amniotic cells. The analysis of these defects has been complicated by lack of knowledge about the activities and distribution of the different enzymes present in normal cells from these sites and how the findings relate to enzyme levels in other tissues. The deficiency is in the lysosomal glycoprotein neuraminidase; by contrast the glycolipid enzyme activities appear to be normal (Cantz, 1982). Strecker & Michalski (1978) suggested that separate enzymes were responsible for hydrolysing (2 $\rightarrow$3) and (2 $\rightarrow$6) linkages and presented evidence in support of the theory that mucolipidosis I was due to a specific deficiency in a (2 $\rightarrow$6) enzyme whereas mucolipidosis II was associated with lack of both enzymes; it now appears more probable, however, that both activities are due to the same glycoprotein enzyme (Cantz, 1982).

In addition to these primary defects a number of other conditions have been found where the neuraminidase deficiency occurs in association with deficiency of other lysosomal enzymes. Some patients have a combined deficiency of neuraminidase and β-galactosidase that may be due to defective post-translational modification of both these enzymes rather than to separate primary enzyme defects (Hoogeveen et al., 1980). The neuraminidase deficiency in patients with mucolipidosis II (I-cell disease) and mucolipidosis III (pseudo-Hurler polydystrophy) is part of a general picture of deficiency of many lysosomal hydrolases, secondary to a general defect in lysosomal enzyme compartmentalisation (Brady, 1982). A partial deficiency of lysosomal ganglioside neuraminidase has been reported in mucolipidosis IV

(Bach, Zeigler & Kohn, 1980) but the biochemical lesion has not yet been fully characterised.

These studies demonstrate the importance of mammalian neuraminidases in catabolism of sialoglycoconjugates and offer prospects for further studies to determine their normal roles in greater detail. Different lysosomal neuraminidases have been shown to be involved in the primary stages of degradation of sialoglycoproteins and of gangliosides and the outline of these degradative processes is now clear (see Suzuki, 1976; Patel, 1978). The overall co-ordination of sialoglycoconjugate synthesis and catabolism and its role in regulating biological processes remain unknown, as do the roles of neuraminidases at other cellular sites or in cell-surface membranes. Presumably, many different types of cell have mechanisms for the controlled turnover of endogenous sialoglycoconjugates; some tissues, e.g. liver, may have further roles in scavenging cells and sialoglycoconjugates from the circulation. Neuraminidase may well have particular roles in brain tissues. The evidence for a variety of enzymes with different properties in various tissues and cell fractions makes it probable that mammalian tissues contain multiple enzymes with a variety of specific roles in sialoglycoconjugate metabolism; this may be contrasted with the broad substrate range of many bacterial neuraminidases, e.g. that from Clostridium perfringens, which may be thought to have a broader digestive function.

VIRAL NEURAMINIDASE

Early studies on the interactions of influenza A virus and red blood cells led to the identification of neuraminidase as a structural component of the virus (see Gottschalk, 1960). It seems probable that this unique virus-coded enzyme has an important biological role and the following section reviews the evidence for this. The general term myxovirus (myxo = mucin) was introduced to embrace other viruses that behave in the same manner as influenza viruses, i.e. with haemagglutinating and neuraminidase activities. The current classification (see Stuart-Harris & Schild, 1976; Ginsberg, 1980) distinguishes the orthomyxoviruses, which comprise influenza viruses A, B and C, and the paramyxoviruses, which include parainfluenza viruses, mumps virus and Newcastle disease virus. All are enveloped single-strand (negative-strand) RNA viruses of similar structure. Measles virus and respiratory syncytial virus are classified with the paramyxoviruses but are less closely related and do not contain neuraminidase.

Ortho- and para-myxoviruses are distinguished by a number of features. The typical oval viral particles of orthomyxoviruses are smaller (diameter 80-120 nm) than those of the paramyxoviruses (150-250 nm); the ribonucleoprotein core is also proportionately smaller. Influenza viruses have a fragmented genome, with separate segments of ribonucleoprotein corresponding with individual viral genes, whereas paramyxoviruses have one long continuous unsegmented genome. The haemagglutinin and neuraminidase are present in separate glycoprotein spikes on the surface of influenza viruses but the two activities are associated with a single subunit in

paramyxoviruses (Scheid & Choppin, 1973). Much less is known about paramyxovirus structure and pathogenesis and the present discussion of viral neuraminidase is based on the influenza viruses.

Influenza A is a major cause of respiratory infection in man; related strains are found in some domestic animals and in a wide range of domestic and wild birds (for a comprehensive monograph on influenza A virus see Stuart-Harris & Schild, 1976). Influenza A causes epidemics which recur every few years in various parts of the world, and which occasionally sweep round the world as pandemics. This fluctuating pattern is associated with changes in the surface antigens, the so-called antigenic shift and drift of influenza A virus. By contrast influenza B and C viruses show less antigenic variation than type A and do not cause epidemics; each is found only in man and no animal reservoir has been identified. Influenza B is responsible for less severe, endemic, respiratory tract infections; influenza C viruses are seldom associated with clinical symptoms and appear to be widespread but relatively avirulent (see Palese & Young, 1982). Influenza C viruses differ in that they lack neuraminidase activity (Kendal, 1974); it is tempting to associate the greater virulence of A and B viruses with their possession of the enzyme. The pathogenicity of influenza viruses has been reviewed by Sweet & Smith (1980); little is known about the determinants of virulence in these viruses but there is genetic evidence that both the haemagglutinin and the neuraminidase are important.

Pandemics of influenza A such as occurred in 1918, 1957 and 1968 are thought to be related to major shifts in antigenic pattern such that antibody produced in response to previously circulating

strains gives very little protection. The haemagglutinin (H) is clearly the most important antigen; anti-H antibody is neutralising and protective. However, there is evidence that the neuraminidase (N) also plays a part and that anti-N confers a significant degree of protection (see below). Influenza A virus was first cultured from swine in 1931, and from man in 1933; since then many isolates have been collected and compared in world-wide surveys. Earlier attempts to classify the major antigenic changes in influenza viruses have been revised in recent years; the following outline is drawn from the discussions in Stuart-Harris & Schild (1976), Ginsberg (1980), Palese & Young (1982) and Webster et al. (1982).

Three major antigenic types of haemagglutinin (H_1, H_2 and H_3) have been identified in human infections, but some nine others have been recognised in animals and birds. There is considerable interest in the possibility that human influenza A viruses may occasionally infect animals and that new H antigens from animal viruses may be picked up by human strains during mixed infection; a number of animal strains have H antigens similar to those of human strains, and there is evidence that the H antigen of the swine virus isolated in 1931 (H_{SW}) had been present in human strains in 1918. Two major N antigens have occurred in human strains, N_1 before and N_2 after 1957; these also occur in avian strains and a further seven N antigens have been found in birds and horses.

The antigenic make-up of strains prevalent between 1947 and 1957 was H_1N_1; the 1957 pandemic resulted from a major shift in both antigens to H_2N_2 and in 1968 there was a further shift to H_3N_2

strains. Serological evidence suggests that the strains prevalent at the end of the 19th century were also H_3N_2 and that the 1918 pandemic was due to $H_{SW}N_1$ strains. The earliest available human isolates, from 1933-47 have been classified as H_0N_1 but it is less certain that a major shift in H antigen occurred in 1947 and they are often now included with the H_1N_1 group.

The appearance of a new pandemic strain has usually been associated with the disappearance of the previous epidemic strain, although the reasons for this are not clear. In 1976 there was a small localised outbreak of influenza in the USA due to a $H_{SW}N_1$ virus. This did not spread and rapidly disappeared again; it may have had an animal origin. The following year saw the reappearance of H_1N_1 strains in China and Russia. They were very similar to strains last seen 20 years previously in man and no animal reservoir has been shown; it is possible that the origin was a laboratory strain or that there are mechanisms by which such strains can lie latent in man for long periods (Laver & Webster, 1979). Since 1977, for the first time in 40 years experience, two antigenic types (H_1N_1 and H_3N_2) have circulated together in the world. It seems that antibody to H_1N_1 strains in the older group of the population has helped to contain this strain and modify the pandemic pattern otherwise expected after a major shift in both antigens. The unpredictability of the antigenic patterns that may emerge presents a very serious problem for the formulation of vaccines that might give effective protection in future pandemics.

Against this background of major antigenic shift there is a constant pattern of minor antigenic drift, an accumulation of small changes in both H and N antigens that gradually produces strains

that are less effectively inhibited by antibodies produced against previously circulating strains and that may then cause local epidemics. The genetic basis of shift and drift was originally deduced from analysis of epidemiological and antigenic changes but has recently been confirmed by detailed structural analyses of representative haemagglutinin and neuraminidase molecules (see Webster et al., 1982). Antigenic drift occurs by accumulation of point mutations affecting the antigenic determinants of the molecules, which are closely related within the whole serological group; shift, however, represents a much more major change that must be due to acquisition of a gene for a more distantly related protein from another strain of virus. The influenza virus genome consists of eight separate ribonucleoprotein segments, each corresponding to a specific viral polypeptide. During experimental mixed viral infections, such recombinants with reassortments of these fragments from the parental strains can be produced.

The structure of influenza A viruses as revealed by electron microscopy was reviewed by Wrigley (1979). Two types of protein spike can be identified on the surface of the viral particles; these are morphologically distinct and correspond to the haemagglutinin and neuraminidase. There is a variable excess of haemagglutinin spikes, usually in the proportion of c. 5 to 1. The structure of the haemagglutinin has been investigated in detail (see Ward, 1981). The gene product is a polypeptide of MW c. 80,000 that is cleaved during maturation into two polypeptides, HA_1 (50,000) and HA_2 (30,000), which remain bound together by disulphide bonds. The haemagglutinin spike is a trimer of this molecule arranged with a large glycosylated hydrophilic portion on

the outside of the host-derived lipid envelope and a hydrophobic portion at the base, inserted into the envelope. The tertiary and quaternary structure of this unusual protein have been resolved in considerable detail (Waterfield et al., 1979; Wilson, Skehel & Wiley, 1981; Webster et al., 1982). There are highly conserved amino-acid sequences in a pocket at the end of the spike that is thought to be the NANA-binding site. The antigenic sites are in variable sequences on loops of the polypeptide chain exposed in the superficial globular part of the spike (Wiley, Wilson & Skehel, 1981). Drift and shift are reflected in minor and major sequence differences in these regions of the polypeptide; a dendrogram charting the degree of relatedness within and between H-antigen groups was given by Webster et al. (1982).

Similar information is now available for the N antigens. The neuraminidase spikes are mushroom-shaped on electron microscopy, with the slender stalk inserted into the membrane (Wrigley, 1979). Each spike is a tetramer of the gene product, an uncleaved polypeptide of MW c. 60,000. Amino acid sequences have been determined for the enzyme from several strains (Fields, Winter & Brownlee, 1981) and the three-dimensional structure of the spike has recently been reported (Jackson & Webster, 1982; Varghese, Laver & Colman, 1983). The neuraminidase, like the haemagglutinin, is a transmembrane glycoprotein with a short hydrophilic tail beyond the hydrophobic intramembrane portion; the slender glycosylated stem is relatively unfolded and flexible. The catalytic sites are located on the upper corners of the box-shaped tetramer that forms the head of the spike; the antigenic determinants are in variable loops that form a nearly continuous surface across the top of the

subunit, encircling the catalytic sites (Colman, Varghese & Laver, 1983). Comparison of amino acid sequences of variants between 1968 and 1975 confirmed the clustering of point mutations in these sites during this period of antigenic drift. It is clear that the haemagglutinin and the neuraminidase of influenza A virus have very different structures and that the NANA-recognising pockets on the two subunits are also dissimilar; this runs counter to an earlier theory that the two molecules might have a common evolutionary origin (Gottschalk, 1960; Wiley, 1983).

The properties of viral neuraminidases have been reviewed by Drzeniek (1972), Rosenberg & Schengrund (1976b) and Schauer (1982). The enzyme spikes can be released from the viral particles by detergent disruption of the lipid envelope or by protease digestion of the subunit stalk, and pure preparations are readily produced. In general, viral neuraminidase is much more active against (2 → 3) linked substrates than the (2 → 6) forms; this can be used in analysis of sialic acid linkage under carefully standardised conditions when only the (2 → 3) linkage is hydrolysed (Gottschalk & Drzeniek, 1972). Neuraminidases from different myxoviruses vary greatly in their ability to hydrolyse (2 → 8) linkages (Corfield & Schauer, 1982b). The pH optimum, temperature optimum and Km values, as with bacterial neuraminidases, may vary with different substrates, buffers and ionic strengths; the pH optimum is usually in the range 4.5–6.5. Most viral neuraminidases are strongly Ca^{2+} dependent.

Biological role of viral neuraminidase

The replication cycle of influenza virus in cell cultures was reviewed by Stuart-Harris & Schild (1976) and Ginsberg (1980). The roles of the haemagglutinin and the neuraminidase have been investigated in a variety of ways, including the use of specific antisera prepared against each subunit. It appears that they have inter-related roles, with the haemagglutinin primarily involved in initiation of infection whereas the neuraminidase is more important in viral release and spread. The haemagglutinin is responsible for initial attachment of the virus to sialic-acid-containing molecules (receptors) on the host-cell surface; anti-H antibody neutralises the virus, preventing attachment and infection. Neuraminidase is not thought to play a direct role in this process as anti-N does not interfere with infectivity.

After adsorption the viral ribonucleoprotein enters the cell cytoplasm, although the detailed process by which initial binding of NANA receptors by the tips of the haemagglutinin spikes leads to fusion of the viral envelope with the cell membrane remains unclear (see Kaariainen & Pesonen, 1982). When influenza virus binds to red cells there is no fusion or infection and the virus soon elutes again from the cells as neuraminidase splits the NANA from the cell-surface glycoconjugates, destroying the receptors. When virus adsorbs to susceptible host cells, fusion occurs before neuraminidase action can elute the particles again. The relative proportions of the two viral subunits and their relative affinities for particular types of surface sialoglycoconjugate presumably determine the balance between binding and elution; this is

reflected in the classic studies demonstrating a receptor gradient on red cells for different types of myxovirus (Burnet et al., 1946; Ginsberg, 1980). A similar process is thought to occur in natural infection when influenza virus encounters glycoproteins in the mucus that overlies the respiratory epithelial cells; after initial unproductive binding the virus is released again. It appears that this is one of the major roles of the viral enzyme, enabling the particles to penetrate the protective mucus layer that lies between the inhaled virus and the target epithelial cells (Gottschalk & Bhargava, 1971).

Neuraminidase is not thought to have an essential role during intracellular replication of myxoviruses (see Drzeniek, 1972); influenza C virus and a number of paramyxoviruses either lack or have very weak neuraminidase activity, and neuraminidase-defective mutants of influenza A virus have been isolated (Palese et al., 1974). It may be, however, that neuraminidase is a virulence factor for influenza A virus, processing viral glycoproteins by preventing addition of sialic acid during their production in the infected cell. Nakajima & Sugiura (1980) demonstrated that in at least certain experimental infections this action is required in order to expose the site for proteolytic cleavage of haemagglutinin to HA_1 and HA_2, a step that is essential for infectivity. Palese et al. (1974) suggested that if viral glycoproteins are not desialylated before viral release there is aggregation of the progeny virus due to interaction of haemagglutinin with NANA on other virions.

A number of lines of evidence have suggested that neuraminidase is important in viral release and spread and that

anti-N is protective at this point in the replication cycle. During infection haemagglutinin and neuraminidase subunits are produced and inserted into localised areas of host-cell membrane; the mature virus is produced by budding, the ribonucleoprotein core picking up its envelope complete with spikes as it passes through the altered cell surface. Rosenberg & Schengrund (1976b) suggested that the localised removal of sialic acid from these areas of host-cell membrane may alter the physico-chemical properties of the membrane so as to permit budding and viral release. Removal of cell-surface sialic acid is also important to prevent emerging virus from readsorbing to the host cell. The effect of specific anti-N on replication of influenza virus was investigated by Seto & Rott (1966) and by Dowdle, Downie & Laver (1974); the number of plaques was not affected but the size of the plaques was markedly reduced, suggesting interference with viral release. This was initially ascribed to inhibition of enzyme activity at the cell surface but later studies suggested that this might not be the only mechanism. Divalent antibody may limit viral release by aggregating viral particles or by bridging between neuraminidase subunits in the emerging virus and in the infected host-cell membrane; univalent Fab fragments that could inhibit the enzyme activity did not reduce virus release (Becht, Hammerling & Rott, 1971).

Antibody to both haemagglutinin and neuraminidase is easily produced in man and in laboratory animals in response to natural or experimental infection or to administration of killed whole virus or purified subunit vaccines. The viral enzyme is a good antigen, perhaps because of its particulate form in the viral envelope.

Anti-N may be detected by its ability to inhibit enzyme activity, though immunodiffusion techniques may be more convenient (see Stuart-Harris & Schild, 1976). The degree of inhibition of the enzyme by antibody depends on the test substrate; anti-N gives complete inhibition with high-MW glycoproteins but only partial inhibition with smaller glycopeptides and an insignificant effect with sialyl-lactose as substrate (Fazekas de St Groth, 1963). The structural data reviewed above confirm that the catalytic site is distinct from the antigenic determinants, so that inhibition is by steric hindrance and is more efficient with larger substrate molecules.

Most studies have used serum antibody levels in assessing the immune response to influenza viruses, and there is good correlation between these and immunity. It may be argued that secretory antibody in respiratory tract mucus, perhaps largely IgA, is more likely to mediate the protective effect in this disease where virus remains localised and very seldom spreads to blood or other tissues; however, secretory antibody is much more difficult to study directly and may often correlate reasonably with serum antibody responses. Although there is evidence that cell-mediated immunity is important in experimental mouse infection (Virelizier, Allison & Schild, 1979) the importance of this aspect of the immune response in natural human infection remains in some doubt (Potter & Oxford, 1979).

The availability of sera with high specific anti-N activity but lacking anti-H effective against a particular challenge strain of influenza virus allowed assessment of its protective effect in experimental infections. Schulman, Khakpour & Kilbourne (1968)

showed passive protection by anti-N in mice, and Rott, Becht & Orlich (1974) confirmed this in chicks. Various studies have shown the protective effect of anti-N for experimental infection in human volunteers (e.g. Murphy, Kasel & Chanock, 1972; Couch et al., 1974). A protective effect during natural infection was demonstrated by Monto & Kendal (1973) who related attack rates with the new H_3N_2 pandemic strain to the presence or absence of serum anti-N_2 produced by previous exposure to H_2N_2 infection. It is now generally accepted that anti-N does give useful protection in man and both haemagglutinin and neuraminidase are included in most human vaccines (see Tyrrell & Smith, 1979). A case has also been made for the use of purified neuraminidase subunit vaccine in order to modify subsequent natural infection and produce long-lasting immunity to both antigens (Couch et al., 1974; Arora, 1979).

Thus there is no doubt of the importance of neuraminidase in the natural history of influenza A virus and of anti-neuraminidase in helping to limit its spread. The enzyme probably has a direct role in hydrolysing glycoproteins and helping the virus to penetrate protective mucus layers. The viruses have evolved a specific adhesion mechanism, the haemagglutinin, that greatly increases their ability to bind to and infect the target cells in the host's respiratory tract epithelium. However, this mechanism also brings disadvantages because it would lead to non-specific binding to NANA residues present on mucus glycoproteins, etc., and without the enzyme this would lead to enhanced trapping by non-specific inhibitors. Similarly, during release of the virus the haemagglutinin would tend to readsorb to NANA residues on the host-cell surface, with reduced viral dispersal. For dissemination

from the host the virus must not only be able to spread locally to other cells in the upper and lower respiratory tract but must also be able to penetrate the mucus and be dispersed in active form in respiratory droplets; here again the enzyme is required to prevent neutralisation of the virus by adsorbed sialoglycoprotein.

There are some parallels between these processes in influenza virus infection and those involved with bacteriophage infection of capsulate host strains. In a number of cases specific enzymes able to hydrolyse the capsular polysaccharide have been shown to be associated with the bacteriophage tail and to facilitate penetration and release. The presence of neuraminidase has recently been demonstrated in two bacteriophages that infect strains of Escherichia coli of capsular type K1; this capsular material is colominic acid, the (2 → 8) linked polymer of NANA, and the bacteriophage enzyme is unusual in giving good hydrolysis of this material (Kwiatkowski et al., 1983).

BACTERIAL NEURAMINIDASE

Bacterial neuraminidase was initially detected during investigations of the interactions of influenza viruses with red blood cells by Burnet and co-workers in the 1940's. The enzyme was first demonstrated in culture supernates of *Clostridium perfringens* and *Vibrio cholerae* (Burnet *et al.*, 1946; Burnet & Stone, 1947; McCrea, 1947); these remain the most intensively studied and widely used bacterial neuraminidases. The production of the enzyme by various other micro-organisms was reviewed by Drzeniek (1972) and Muller (1974a); table Intro/V lists species representative of the main groups of neuraminidase-producing organisms, which include cocci and bacilli, aerobes and anaerobes, Gram-positive and Gram-negative species. It should be appreciated that in many cases only a small number of strains have been examined and that in some cases only a proportion of these could be shown to produce the enzyme.

Several different groups of Gram-positive bacteria have been shown to contain neuraminidase-positive species. Chu (1948) reported receptor-destroying enzyme activity in *Streptococcus pneumoniae*; the neuraminidase was later characterised and shown to be produced by many clinical isolates (Kelly, Greiff & Farmer, 1966; Kelly, Farmer & Greiff, 1967). Strains from a variety of other streptococcal species also produce neuraminidase, including various oral strains of the viridans group, e.g. of *Streptococcus sanguis* and *Streptococcus mitis* (Laurell, 1959; Hayano & Tanaka, 1969; Hayano, Tanaka & Okuyama, 1969). Only a few strains of Lancefield group-A streptococci produce the enzyme (Davis, Baig & Ayoub, 1979). Clinical isolates of group-B streptococci may also

TABLE Intro/V

Neuraminidase-producing micro-organisms

Type of micro-organism	Representative neuraminidase-positive species[a]
Bacteria	
(a) Gram-positive	*Streptococcus pneumoniae*
	Bifidobacterium bifidum
	Clostridium perfringens
	Corynebacterium diphtheriae
	Propionibacterium acnes
	Arthrobacter ureafaciens
	Erysipelothrix insidiosa
(b) Gram-negative	*Vibrio cholerae*
	Campylobacter foetus
	Pasteurella multocida
	Haemophilus parainfluenzae
	Klebsiella aerogenes
	Bacteroides fragilis
	Capnocytophaga ochracea
Mycoplasma	*Mycoplasma gallisepticum* (?)
Actinomycetes	*Streptomyces albus*
Fungi	*Sporotrichum schencki*
	Penicillium urticae
Protozoa	*Trichomonas foetus*
	Trypanosoma cruzi

[a] For references and fuller details see text.

be neuraminidase-positive (Mattingley et al., 1980); other positive strains have been found in serogroups C, E, G, H, K and L (see Muller, 1974a). The enzyme is also produced by Bifidobacterium bifidum (Lactobacillus bifidus), a commensal found in large numbers in the gut in breast-fed infants, but has not been found with other lactobacilli (Shilo, 1957; Von Nicolai & Zilliken, 1972); these organisms are sometimes grouped with the streptococci in the broader category of Lactobacillaceae.

Many species of clostridia besides Clostridium perfringens produce the enzyme; these are reviewed in the Discussion section. The original investigations of panagglutinability found that diphtheroid strains could produce the Thomsen-Friedenreich phenomenon (Friedenreich, 1930). Later studies (Blumberg & Warren, 1961; Warren & Spearing, 1963) detected neuraminidase activity in crystalline preparations of diphtheria toxin; Moriyama & Barksdale (1967) demonstrated its production in cultures of Corynebacterium diphtheriae and confirmed that it was quite distinct from the toxin. A number of other corynebacterial strains have also been found to produce the enzyme, including Corynebacterium haemolyticum, Corynebacterium pyogenes, Corynebacterium ulcerans, Propionibacterium acnes and commensal diphtheroids now classified as Brevibacterium species (Muller, 1974a; Tisserand-Jochem, 1974; Von Nicolai, Muller & Zilliken, 1975; Vertiev & Ezepchuk, 1981). Various other Gram-positive bacteria may be broadly related to the corynebacteria, whose classification remains difficult. Neuraminidase production has been found with Arthrobacter species (Uchida, Tsukada & Sugimori, 1977; Wang et al., 1978) and with

Erysipelothrix rhusiopathiae (insidiosa) (Muller, 1974c; Von Nicolai, Muller & Zilliken, 1978). Arden, Chang & Barksdale (1972) surveyed a representative range of corynebacteria and related Mycobacterium and Nocardia species but found the enzyme only in the genus Corynebacterium.

Neuraminidase is also produced by several Gram-negative bacterial species. In addition to Vibrio cholerae, a number of so-called non-agglutinable vibrios and some animal strains of Campylobacter (Vibrio) foetus have been reported to produce the enzyme (Morris & Park, 1971; Muller, 1973a, 1974a). A number of early reports using indirect techniques have not been confirmed, e.g. those of Shilo (1957) for various Pseudomonas species and Laurell (1959) for Pasteurella pseudotuberculosis. However, the enzyme has been clearly demonstrated in Pasteurella multocida and Pasteurella haemolytica (Scharmann, Drzeniek & Blobel, 1970). Oral strains of Haemophilus influenzae and Haemophilus parainfluenzae have been found to produce neuraminidase (Tuyau & Sims, 1974) though it has not been reported in strains causing systemic infections (O`Toole, Goode & Howe, 1971). Amongst the Enterobacteriaceae it has been detected only in Klebsiella aerogenes (Pardoe, 1970). Muller & Werner (1970b) described neuraminidase production by Bacteroides fragilis and some other strictly anaerobic species of Gram-negative bacilli; this group of organisms is reviewed in the Discussion section.

A number of other microbial sources of the enzyme have been reported. Several Mycoplasma species adsorb to NANA-containing receptors on host cells but only Mycoplasma gallisepticum has been reported to produce a neuraminidase (Sethi & Muller, 1972); this

might imply parallels with myxoviruses, although later workers have failed to confirm the original report (Glasgow & Hill, 1980). The enzyme has been detected in cultures of a few species of Streptomyces (Myhill & Cook, 1972; Kunimoto et al., 1974) and also in some eukaryotic fungal species of Sporotrichum and Penicillium (Uchida, Tsukada & Sugimori, 1974). Neuraminidase production has also been reported in the protozoal pathogens Trichomonas foetus (Watkins & Morgan, 1954; Romanovska & Watkins, 1963) and Trypanosoma cruzi (Muller, 1974a).

A number of reviews have covered various aspects of bacterial neuraminidases (Rafelson, Schneir & Wilson, 1966; Gottschalk & Bhargava, 1971; Drzeniek, 1972, 1973; Gottschalk & Drzeniek, 1972; Rosenberg & Schengrund, 1976b; Corfield & Schauer, 1982b; Schauer, 1982). The present account is limited to a general outline; a fuller assessment of the neuraminidases produced by anaerobic bacteria and their possible role in pathogenesis is reserved for the Discussion section.

Neuraminidase remains cell-associated in some bacterial species, e.g. Corynebacterium diphtheriae (Moriyama & Barksdale, 1967) and Pasteurella multocida (Drzeniek, Scharmann & Balke, 1972), but there may be variable release into the culture medium with a number of bacteria; the high levels of exoenzyme production found with Vibrio cholerae (Rosenberg, Binnie & Chargaff, 1960), Clostridium perfringens (Cassidy, Jourdian & Roseman, 1966) and pneumococci (Kelly et al., 1966) have provided the most convenient starting material for preparation and purification of the enzyme. Enzyme production depends on the culture medium and in a number of

cases has been found to be inducible when various substrate preparations or sialic acid derivatives are added to a basal medium that lacks glucose (see Pardoe, 1974). This has been shown for Vibrio cholerae (Ada & French, 1959; French & Ada, 1959), pneumococci (Kelly et al., 1966), Pasteurella multocida (Drzeniek et al., 1972), Klebsiella aerogenes (Pardoe, 1970), Clostridium perfringens (Nees & Schauer, 1974a) and Arthrobacter sialophilus (Wang et al., 1978).

The split product of neuraminidase activity, NANA, may tend to inhibit the enzyme's activity but it may also induce production of N-acylneuraminate pyruvate-lyase (NAN-lyase; EC 4.1.3.3), an enzyme that degrades free NANA to pyruvate and N-acetyl mannosamine; the latter may act to induce neuraminidase production in some bacterial species so that production of both enzymes is co-ordinated (see Pardoe, 1974; Rosenberg & Schengrund, 1976b). The Clostridium perfringens NAN-lyase has been most intensively studied (Comb & Roseman, 1960; Brunetti, Swanson & Roseman, 1963; Nees et al., 1976) but it has also been detected in many other neuraminidase-producing organisms (Arden et al., 1972; Drzeniek et al., 1972; Muller, 1974a). NAN-lyase activity is also found in various mammalian tissues, where it may have a regulatory role in sialic acid metabolism; the reaction is reversible but a catabolic role is favoured (Corfield & Schauer, 1982b). The combination of neuraminidase and NAN-lyase is thought to enable bacteria not only to degrade sialoglycoconjugates but also to take up the released NANA and use its constituents as a source of energy (Schauer, 1982).

Methods for purification of bacterial neuraminidases have been reviewed by Rosenberg & Schengrund (1976b) and Wadstrom (1978); it should be appreciated that purification and characterisation has been undertaken with only a few of the bacterial species listed above. In general, conventional methods of protein purification have been used, usually with ammonium sulphate fractionation followed by chromatographic separation, but this often gives preparations that are still contaminated with other bacterial enzymes or toxins (Kraemer, 1968; Den, Malinzak & Rosenberg, 1975). More recent techniques such as affinity chromatography, polyacrylamide gel electrophoresis and isoelectric focusing have yielded very pure preparations (see Rosenberg & Schengrund, 1976b; Schauer, 1982).

Determinations of MW of bacterial neuraminidases have usually given results between 50,000 and 90,000 (see Wadstrom, 1978). In a number of cases there is evidence for multiple forms of the enzyme with different MW, perhaps partly attributable to proteolytic digestion (Rosenberg et al., 1960; Rood & Wilkinson, 1976a; Uchida, Tsukada & Sugimori, 1979; Corfield & Schauer, 1982b); no definite evidence for subunit structure is available. A number of "isoenzymes" of similar MW but different charge were found in studies of pneumococcal neuraminidase (Tanenbaum & Sun, 1971); microheterogeneity of neuraminidase molecules was also detected by Moriyama & Barksdale (1967) and Nees et al. (1975).

The pH optimum of a particular neuraminidase may vary considerably when tested with different substrates (Rafelson, Schneir & Wilson, 1963; Schauer, 1982), with different buffer ions (Cassidy et al., 1966) or at different ionic strengths

(Barton, Vaskresenija & Rosenberg, 1975). Optimal pH values quoted for bacterial neuraminidases are usually in the range 4.5-6.5 (Drzeniek, 1972). Neuraminidases may also vary in their calcium dependence and sensitivity to EDTA (Boschman & Jacobs, 1965; Drzeniek, 1972); the enzyme produced by Vibrio cholerae is very calcium-dependent but most other bacterial neuraminidases are not markedly so.

The specificity of various bacterial neuraminidases was reinvestigated by Schauer (1982) using a range of well-defined substrates. Glycoconjugates containing N-glycolyl sialic acids are hydrolysed less well than N-acetyl forms, and the presence of the 4-O-acetyl group markedly reduces hydrolysis by most neuraminidases. Most bacterial enzymes have a broad range of activity and are able to hydrolyse (2→3), (2→6) and (2→8) linkages in oligosaccharide and glycoprotein substrates. Generally, bacterial neuraminidases have greatest activity with (2→3) and least with (2→8) linkages but a number of bacterial enzymes have been found with different affinities, e.g. the Arthrobacter ureafaciens enzyme is most active against the (2→6) linkage (Uchida et al., 1979). Bacterial enzymes can remove the external NANA residues from gangliosides; the internal NANA is resistant to Vibrio cholerae and most other bacterial neuraminidases but can be slowly hydrolysed by the Clostridium perfringens enzyme in the presence of bile salts (Gatt, Gazit & Barenholz, 1981). The broader substrate range of bacterial neuraminidases may be related to their occurrence as soluble exoenzymes of relatively low MW in contrast to the higher MW of the tetrameric viral enzyme subunits and the particulate membrane-associated mammalian enzymes.

Biological role of bacterial neuraminidase

The biological role of bacterial neuraminidase is not fully understood but it seems probable that it is basically a digestive and nutritional enzyme. It is unlikely that it has a primary intracellular role in normal bacterial metabolism as the enzyme occurs much more widely than do sialic acids, which are relatively uncommon in bacterial species. It is often associated with NAN-lyase, although either enzyme may occur alone in various species (Arden et al., 1972; Muller, 1974a). Production of both enzymes allows release of sialic acids from sialoglycoconjugates and subsequent use of the constituents as a source of energy; Schauer (1982) discussed the co-ordination and induction of this pathway, which may also require a permease for uptake of NANA since NAN-lyase is an intracellular enzyme. Even in species where neuraminidase is predominantly cell-associated it appears that at least a proportion of the enzyme is available on the cell surface to interact with exogenous substrate (Pardoe, 1974).

The broad substrate specificity of most bacterial neuraminidases also tends to support a general role as a digestive enzyme. Removal of terminal NANA residues is an important first step in the degradation of glycoproteins and glycolipids; in some cases neuraminidase treatment may be necessary before protease can attack glycoproteins (Schauer, 1982). A number of neuraminidase-producing bacteria, e.g. streptococci and clostridia, produce a range of other extracellular enzymes that can degrade glycoproteins or attack cell-surface constituents, e.g. protease, hyaluronidase, phospholipase and various glycosidases; it seems probable that

neuraminidase may be a useful addition to this family of digestive enzymes and will play a role in nutrition of the bacteria in various natural environments.

As previously outlined, substrates for neuraminidase action are common in nature, being widespread in vertebrates and higher invertebrates although not occurring in lower eukaryotes, plants and most prokaryotes. Saprophytic bacteria have an important scavenging role and neuraminidase is likely to be of assistance in breaking down mucous secretions, faecal material and dead tissues. Some saprophytic organisms live primarily in soil but others are also commensals of higher animals, colonising epithelial surfaces and being constantly shed from the body to the environment along with dying cells, faeces, etc. Commensal organisms live in a symbiotic association with the host, having a protected environment and deriving nutrition from it, sometimes carrying out biochemical transformations of value to their host, and helping to exclude more pathogenic species from various ecological niches in the host. The distinction between commensal and pathogen is a fine one and many bacteria may be on either side of the boundary, depending on the biochemical attributes of particular bacterial strains and on the defence mechanisms of the individual host. At death, the commensal flora are no longer controlled and play their part as saprophytes in digesting the tissues of the corpse.

The list of neuraminidase-producing organisms (see table Intro/V) contains many bacteria that are commensals of man and animals; even such well-known pathogens as Clostridium perfringens, Vibrio cholerae and Corynebacterium diphtheriae may also have a commensal role or be associated with long-term symptomless

carriage. A variety of species of clostridia, corynebacteria, streptococci, bifidobacteria and bacteroides organisms are prominent in the commensal flora of human epithelial surfaces, on the skin, in the mouth, throat, gut or vagina. In all these sites there are sialoglycoconjugates in mucous secretions and on epithelial cell surfaces and it seems probable that neuraminidase-producing bacteria will play a part in their breakdown (Gottschalk, 1960; Collee, 1965b; Muller, 1974a).

The lower gut in particular contains large numbers of bacteria that thrive on the nutrients available in the dietary debris, the intestinal secretions and the large number of cells that are shed daily from the intestinal epithelium. The absorptive power of the lower gut is limited and most of the nutrients reaching the colon are utilised by the bacterial flora. Hoskings (1978) discussed the role of bacteria in degradation of glycoproteins in saliva and intestinal secretions. Studies with germ-free rats confirmed that bacterial glycosidases are required for degradation of the oligosaccharide portion of the intestinal mucus, although host mechanisms may be largely responsible for proteolysis. Neuraminidase activity in the gut and saliva is almost entirely contributed by bacteria; a considerable amount of sialoglycoconjugate remained in the faeces of germ-free animals whereas almost none was detectable in normal faeces.

When this broad picture is considered it is not surprising that a number of saprophytic and commensal bacteria can produce neuraminidase as a digestive and nutritional enzyme; medical interest in bacterial neuraminidase derives from the possibility

that the enzyme may also be a virulence factor for some pathogenic species, contributing to damage of host tissues or the evasion of host defences even though it is not itself directly toxic. For many years the main advances in analysing bacterial pathogenesis lay in the detection and characterisation of specific toxins that could reproduce the main features of clinical disease. This approach has had many successes, e.g. with diphtheria, tetanus and cholera toxins, but it is apparent that even in these diseases other factors are also important, while there are many diseases where the damage to the host cannot be attributed to individual toxic products.

Possible roles of neuraminidase in bacterial pathogenicity

Pathogenicity is the result of interaction between micro-organisms and the host. Both sides of the equation are important; the majority of infections occur in people who are in some way compromised - the old, the very young, those with previous injury or surgery, tumours, immunosuppression, etc. Only a proportion of those exposed to bacterial challenge develop clinical infection and the severity of infection varies considerably; this reflects differences in the individual's specific and non-specific defence mechanisms as well as factors in the challenge organism. A few bacteria are clearly virulent organisms capable of causing severe infection in healthy people but many also have a commensal role and only occasionally invade and cause disease. Related strains and species of bacteria can be shown to be of different virulence in experimental models of infection. Some bacteria are almost

incapable of producing infection. The factors that distinguish pathogens and potential pathogens from non-virulent organisms are of great interest as the identification of such virulence determinants may suggest additional therapeutic strategies for control of infection. It seems probable that neuraminidase will continue to play a digestive and nutritional role for bacteria that can invade the tissues and produce disease; it is less certain whether it may be a virulence determinant contributing to the ability of particular bacteria to cause infection.

The broad picture of bacterial pathogenesis has been discussed by Smith (1978) and Mims (1982). Pathogenicity can seldom be understood in terms of a single virulence factor as different factors are likely to be important at different stages in the process of infection. Successful pathogens have to enter the host and reach the target area; bacterial adhesion and other mechanisms of localisation are the subject of much current interest (see Arbuthnott & Smyth, 1979; Ofek & Beachey, 1980). Many bacteria have to be able to evade the host's non-specific defence mechanisms by penetration of protective mucus and inhibition of phagocytosis or intracellular killing; bacterial capsules and other surface components may be important for these processes (Smith, 1977). Pathogenic bacteria must be able to obtain nutrients in or on the host tissues and to compete for essential factors, e.g. iron (Bullen, Rogers & Griffiths, 1978). Damage to the host tissues may be direct, by toxins and exoenzymes, or indirect, leading to damage by inflammation, hypersensitivity or autoimmune reactions (see Arbuthnott, 1978a; Stephen & Pietrowski, 1981). In chronic infections, pathogenic organisms may require mechanisms for

avoiding elimination by the host's specific humoral or cell-mediated immune responses.

Neuraminidase is produced *in vitro* by a number of important pathogenic bacteria and it might be postulated that it plays a role in some of these processes if it is also produced *in vivo* during infection. Pardoe (1974) showed that the enzyme can be induced *in vitro* by a wide range of sialic-acid-containing substances present in mammalian secretions and tissues, and argued that this also occurs *in vivo*. The presence of bacterial neuraminidase in blood or tissues during natural or experimental infection has been demonstrated directly on a number of occasions (e.g. Gadalla & Collee, 1968; Seger *et al.*, 1980; Hof & Loegering, 1982; Schauer, 1982), although the difficulties in distinguishing bacterial and mammalian neuraminidase with confidence must be borne in mind. Production of the enzyme *in vivo* has also been deduced by the demonstration of alterations attributable to neuraminidase in serum or exudate proteins during infection (e.g. Muller, 1970b, 1974a; Muller & Werner, 1970a) or by demonstration of exposed T antigen on red blood cells or in other tissues (Klein *et al.*, 1977; Seger *et al.*, 1980; Seges *et al.*, 1981).

The original evidence for the importance of neuraminidase in infection came from analyses of its role in myxovirus infection (see Drzeniek, 1972). It was initially thought that the production of both haemagglutinins and neuraminidases by some bacterial species might indicate similarities with the process of influenza virus infection. The occurrence of bacterial haemagglutinins was reviewed by Neter (1956); it soon became clear that they might be

either structural or soluble factors (Duguid et al., 1955). The structural haemagglutinins of various bacteria are common fimbriae or related filamentous structures such as the K88 antigen of Escherichia coli (see Duguid & Old, 1980); such surface structures may play an important role in bacterial adhesion to their target cells but the receptors are different from the NANA-containing receptors for myxoviruses (see Ofek & Beachey, 1980) and there is no correlation with neuraminidase production. Soluble haemagglutinins are bacterial products that can adhere to red cells and alter their sedimentation pattern. In general there is little correlation between the production of soluble haemagglutinins and neuraminidase by bacteria (Muller, 1974a; Tuyau & Sims, 1974) and their biological significance remains in doubt (Tavendale et al., 1983).

The closest parallel has been found with clostridial haemagglutinins. Collee (1961, 1962, 1965a and b) was able to distinguish the haemagglutinin of Clostridium perfringens from the phospholipase-C and most of the other soluble products of the organism. It was very closely associated with the neuraminidase, although not identical; freshly isolated neuraminidase-positive strains were haemagglutinin-negative. He concluded that the haemagglutinin may be an inactive form of the enzyme. Similar findings were made for the haemagglutinin and neuraminidase of Clostridium septicum although with this species the haemagglutinin was also produced by freshly isolated strains (Gadalla & Collee, 1967, 1968). The relationship between the haemagglutinin and neuraminidase of Clostridium perfringens was further investigated by Rood & Wilkinson (1975, 1976a and b). Mutants defective in

haemagglutinin production were also defective for neuraminidase; a revertant that regained haemagglutinin production also regained enzyme production. These workers purified the enzyme and found at least two apparently unrelated molecular species; chromatographic methods failed to separate the haemagglutinating and enzymic activities. These studies provide further genetic evidence that the haemagglutinin is likely to be an altered form of the enzyme with affinity for erythrocyte receptors but unable to complete its enzymic action (see Wadstrom, 1978); studies by Tatsuki _et al._ (1981) with a different strain of _C. perfringens_, however, did not confirm that production of the two products was regulated by a common genetic mechanism.

There is no evidence that clostridial haemagglutinin plays a part in bacterial adhesion _in vivo_ and there are obvious differences between the situation with these bacteria and with influenza viruses. It is unlikely that bacterial neuraminidase will play a role similar to that of viral neuraminidase in the complex inter-relationship outlined above where the enzyme appears to be necessary to compensate for the presence of a powerful attachment factor. However, bacterial neuraminidase may well have a comparable role in attacking protective mucous layers and reducing viscosity, preventing bacterial entrapment and allowing access to target cells for bacteria or their toxins. As Gottschalk (1960, p. 102) suggested, the enzyme may have evolved "as part of a vital mechanism to guard the organism against separation from its environment by layers of mucin covering its surface".

It appears that mammalian neuraminidase is predominantly an intracellular enzyme, with carefully regulated activity required for the controlled metabolism of sialoglycoconjugates. The introduction of exogenous bacterial neuraminidase during infection is likely to hydrolyse NANA residues from important circulating glycoproteins and cell-surface components, which may profoundly affect their function, distribution and lifespan in the body. Studies of the effect of injecting bacterial neuraminidases into experimental animals have not shown it to have direct toxic effects (e.g. Burton, 1963) unless large amounts are given, when effects on plasma proteins, red cells and thrombocytes can be shown (Ejby-Poulsen, 1954b; Choi et al., 1972; Gregoriadis et al., 1974; Ronnenberger, 1974). Although neuraminidase is not a classical bacterial toxin, it might be an aggressin, contributing to the pathogenic effect produced by other bacterial factors, and it might occasionally produce the haemolytic or coagulation disorders that may be late complications in a number of severe infections (Seges et al., 1981).

The general proposition that neuraminidase plays a role in bacterial pathogenesis has been supported by two main lines of argument. Muller (1974a) claimed that there is a strong correlation between pathogenicity and neuraminidase production. He argued that in several groups of bacteria the enzyme was produced by the more virulent strains and species, whereas less virulent commensals produced much lower levels or failed to produce it at all. He suggested that the enzyme was commonly inducible in pathogenic bacteria but less so in commensal strains; the ability to boost production of neuraminidase to high levels in vivo would

be a virulence determinant for such strains. Furthermore he argued that the enzyme produced by pathogenic bacteria was more active, with a wider range of substrate specificity, and that this explained the greater virulence of these strains.

The other main line of argument has been the enumeration of biological effects of the enzyme in various test systems. Rosenberg & Schengrund (1976b) listed 123 reports of the effect of neuraminidase on a wide range of glycoconjugates, circulating blood cells and normal or abnormal tissue cells, and argued that such findings, though incomplete, "are suggestive of a role for bacterial sialidase in the aggressive survival of bacterial pathogens". Many such examples of possible effects of exogenous neuraminidase produced during infection have been indicated in this Introductory Review; the possible role of the enzyme in the pathogenicity of particular groups of bacteria is considered in more detail in the following pages.

Pathogenicity of various neuraminidase-producing bacteria

Pneumococci. Pneumococcal strains are commonly present as commensals in the nasopharynx but may become involved in lower respiratory tract infection in people with chronic respiratory disorders or following viral infections. Some strains are more virulent and can produce outbreaks of pneumonia (classically lobar pneumonia) in previously healthy young people. Pneumococci are an important cause of meningitis and can produce serious septicaemia; haemolytic-uraemic syndrome has also been associated with pneumococcal infection. The pathogenesis of pneumococcal

infection remains poorly understood; no conventional exotoxins have been demonstrated and attention has focused on neuraminidase as a probable toxic factor. Kelly and his colleagues (Kelly & Greiff, 1970; Kelly et al., 1966, 1967) found that the enzyme was produced by all of 77 recent clinical isolates although it was lacking in several laboratory-adapted strains. Sterile extracts of neuraminidase-producing strains proved lethal on intraperitoneal injection into weanling mice; the toxicity was related to the neuraminidase content. They further demonstrated that purified neuraminidase from these cell extracts was lethal when given either by the intraperitoneal route or by direct intracerebral injection.

The enzyme has been shown to be produced during experimental infections (e.g. Ejby-Poulsen, 1954a), and there is good evidence for its production and activity during severe human infections, in meningitis (Muller, 1970c; O'Toole et al., 1971; Vierbuchen & Klein, 1983) and also in fatal haemolytic-uraemic syndrome, when T-antigen exposure has been shown both on red cells and in the glomeruli (Klein et al., 1977; Seger et al., 1980). The detailed mechanism of action of neuraminidase in pneumococcal respiratory tract infection remains unclear although it seems probable that it will play a part in combating the normal protective role of respiratory mucus. The role of the enzyme in meningitis is also uncertain. The enzyme can be detected in the CSF in pneumococcal but not meningococcal or Haemophilus meningitis and it has been suggested that its presence may correlate with coma and brain damage (O'Toole et al., 1971); however, no effect on brain gangliosides could be shown in experimental pneumococcal meningitis in dogs (Carruthers & Kanokvechayant, 1973). Vierbuchen & Klein

(1983) demonstrated T-antigen exposure in the choroid plexus in three fatal cases of pneumococcal meningitis and attributed this to bacterial enzyme in the CSF; adsorption of natural anti-T to the altered choroid plexus endothelium might contribute to the pathology. In haemolytic-uraemic syndrome the exposure of T antigen on red cells, thrombocytes and renal glomeruli is postulated to lead to adsorption of anti-T, haemolysis, glomerular damage and the other features of the disease (Klein et al., 1977; Seger et al., 1980).

Other factors are also of importance in pneumococcal infection; Kelly & Greiff (1970) observed that a non-neuraminidase-producing pneumococcal strain was also highly virulent for mice although the cell extract from this strain was non-toxic. The critical importance of the pneumococcal capsule has been recognised for many years; the capsular material itself is non-toxic but it protects the organism from phagocytosis (see Smith, 1977). Nevertheless it is generally accepted that neuraminidase does contribute to the pathogenicity of the organism and is a significant virulence factor in severe pneumococcal infections.

Other streptococci. Muller (1974b) found high levels of neuraminidase activity in isolates of Streptococcus sanguis and other viridans streptococci isolated from patients with bacterial endocarditis. The enzyme was shown to have been active in vivo, producing characteristic changes in the electrophoretic pattern of the serum proteins, and he argued that the virulence of these strains correlated with their ability to produce higher levels of the enzyme than were found with most commensal viridans streptococci.

Neuraminidase is also produced by some strains of group-B streptococci (Streptococcus agalactiae). Milligan et al. (1978) reported that high levels of the enzyme were produced by clinical isolates of type-III group-B streptococci, which are particularly associated with late-onset neonatal infections; interestingly, group-B streptococci also have sialic-acid-containing surface antigens (Baker & Kasper, 1976; Kasper et al., 1979; Doran, Straus & Mattingley, 1980). Mattingley et al. (1980) surveyed a range of type-III strains recently isolated from infected patients and found that the majority, but not all, of the strains did produce the enzyme. Durham et al. (1981), investigating neuraminidase production and virulence of type-III strains in experimental infection of mice, found that although the enzyme was produced by some virulent strains there was no general correlation with virulence. The evidence for an association between virulence and neuraminidase production in group-B streptococci is still incomplete.

Davis et al. (1979) studied laboratory strains of group-A streptococci (Streptococcus pyogenes). Neuraminidase-positive strains were found only in particular serotypes (T1, T4 and T12) that have been associated with production of acute glomerulonephritis, a late complication of Streptococcus pyogenes infection attributed to immune-complex formation. They related their finding to the observation by McIntosh et al. (1972) that serum immunoglobulin treated with neuraminidase from a nephritogenic group-A streptococcus could produce glomerular lesions in experimental animals, and suggested that neuraminidase might be responsible for generating an autoimmune response. However recent studies by

Potter et al. (1982) have not supported a link between neuraminidase and post-streptococcal nephritis; none of 23 strains of T4 and T12 serotypes actually isolated from patients with glomerulonephritis produced the enzyme.

Oral bacteria. There is considerable interest in the oral bacterial flora, partly because of the possible role of various bacterial species in dental caries and gingival infections. Neuraminidase activity in saliva and dental plaque is attributed to their bacterial content (Rogers, Newbrun & Tatevossian, 1979). The enzyme has been shown to be produced by some oral strains of Streptococcus sanguis and Streptococcus mitis (Fukui, Fukui & Moriyama, 1971) and by oral Haemophilus strains (Tuyau & Sims, 1974, 1975) but not by most other oral commensals. At one time it was thought that neuraminidase action on salivary glycoproteins might play a part in their precipitation on teeth and the formation of dental plaque (Leach, 1963). However, no positive correlation between neuraminidase levels and dental caries or periodontal disease has been demonstrated (Perlitsh & Glickman, 1967) and neuraminidase-treated salivary proteins cannot be shown to adhere to teeth (Briscoe, Pruitt & Caldwell, 1972). Attention is now focused primarily on the role of polysaccharides and dextran production by Streptococcus mutans in the build-up of dental plaque (see Gibbons & Van Houte, 1975; McGhee & Michalek, 1981).

Corynebacterium diphtheriae. Neuraminidase production is closely associated with diphtheria toxin production, and commercial antitoxin against diphtheria toxin also contains anti-neuraminidase antibody (Warren & Spearing, 1963). This raised the possibility

that neuraminidase might have been responsible for some of the observed effects of the toxin and that anti-neuraminidase might contribute to the protective effect of the antiserum. However, later studies with purified toxin have very successfully elucidated the molecular mode of action of the toxin, which is clearly responsible for the major features of the disease (see Murphy, 1976).

It remains possible that neuraminidase plays a role in the local establishment of infection in the throat; little is known about this process although successful colonisation must be a prerequisite for the production of toxin and its clinical effects. Ronnenberger (1974) showed that subcutaneous or intramuscular injection of purified Vibrio cholerae neuraminidase into rabbits or guinea pigs produced transient localised inflammatory signs. Ezepchuk & Vertiev (1974) demonstrated similar findings with purified neuraminidase from a non-toxigenic strain of Corynebacterium diphtheriae and claimed that it might be responsible for some of the oedema and spreading of the local lesion in diphtheria. However, diphtheria toxin has marked dermonecrotic activity and, since mass immunisation with diphtheria toxoid has led to virtual disappearance of the organism rather than its transmission in the immunised population as a throat commensal, it seems probable that the toxin itself plays a critical part in the colonisation of the throat and subsequent dissemination. Neuraminidase may play a part in attacking pharyngeal secretions but any more central role in the pathogenesis of diphtheria remains hypothetical.

Vibrio cholerae. The pathophysiology of cholera is now understood in considerable detail but the importance of the large amounts of extracellular neuraminidase that Vibrio cholerae produces remains uncertain. There appears to be a correlation between virulence and neuraminidase production in Vibrio species; large amounts are produced by virulent strains isolated from human infections but much lower levels by environmental strains and non-cholera vibrios (Solovev et al., 1972; Muller, 1973a).

Gastrointestinal infections similar to cholera are produced in young animals and human neonates by strains of Escherichia coli; there are many parallels with cholera but these species do not produce neuraminidase. In each case the organisms penetrate the protective mucous secretions and localise in the upper small bowel, resisting the host's normal defences against bacterial colonisation and the mechanical cleansing produced by peristalsis and the high flow rate of intestinal contents (see Savage, 1980). They then produce enterotoxins, which adsorb to receptors on the epithelial cells; the toxin activates adenylate cyclase inside the cell and switches on the secretion of fluid and electrolytes into the gut that is responsible for the clinical syndrome (see Holmgren, 1981). For enterotoxigenic strains of Escherichia coli, attachment to the intestinal epithelium is mediated by surface bacterial structures such as the K88 antigen found in strains that infect piglets (see Smith & Linggood, 1971; Arbuthnott & Smyth, 1979). Localisation is also essential for Vibrio cholerae infection but occurs by a different mechanism. Freter (1981) has shown that active motility is essential in order to allow the vibrios to penetrate the mucous layer and approach the intestinal villi; it appears that they may

not require to attach to the epithelial surface as do enterotoxigenic Escherichia coli.

Neuraminidase may play a part in this process by reducing the viscosity of the intestinal mucus and assisting penetration by motile vibrios and by toxin. The actual receptor for cholera toxin is GM1 ganglioside on the epithelial cell surface. Studies of neuraminidase action with other cell types showed an increase in the number of receptors for cholera toxin as di- and trigangliosides were hydrolysed to produce more of the monosialoganglioside GM1 (Haksar et al., 1974). It was suggested that cholera neuraminidase might act similarly in the gut but further studies have not been able to show an increased concentration of GM1 receptors following neuraminidase treatment of intestinal epithelium; it appears that there is already an adequate concentration of GM1 in the normal gut (Holmgren, 1981).

Pasteurella species. Muller (1974a) reported changes in the electrophoretic pattern of serum proteins that confirmed the production of neuraminidase during fatal infection of a guinea pig with Pasteurella multocida. Drzeniek et al. (1972) failed to find a correlation between neuraminidase production and virulence of Pasteurella multocida strains for mice. Krasemann & Muller (1974) also found an imperfect correlation between neuraminidase production in vitro and mouse virulence for 25 strains of this organism although some of the most virulent strains produced high levels of the enzyme; they concluded that other factors were important. Frank & Tabatabai (1981) discussed the possible role of neuraminidase in the pathogenicity of Pasteurella haemolytica

strains in sheep and cattle but were unable to demonstrate a good correlation; little is known about the virulence of this species.

Anaerobic bacteria. It is apparent that neuraminidase is produced *in vivo* during infection by a number of significant bacterial pathogens and that it can be expected to act on a variety of important glycoconjugates on mucous surfaces and in the tissues, but persuasive evidence for a role as a critical virulence determinant in the infections reviewed above is still lacking (with the possible exception of the pneumococcus). The experimental work reported in this thesis was designed to investigate neuraminidase production by a variety of species of *Clostridium* and *Bacteroides* and to assess its possible contribution to clostridial myonecrotic infections; the pathogenesis of these infections is discussed in the next section.

CLOSTRIDIAL MYONECROSIS (GAS GANGRENE)

Gas gangrene came into prominence in 1914-18 as an important cause of death due to infection developing after war injury; *Clostridium perfringens* (*C. welchi*), *C. septicum* and *C. novyi* (*C. oedematiens*) are the organisms most commonly involved. The infection is rare in peace-time and most of the literature on clinical and bacteriological aspects comes from military experience in World Wars I and II; comprehensive reviews of clostridial wound infection were provided by Oakley (1954), MacLennan (1962) and Willis (1969).

Classically, the clinical syndrome of gas gangrene develops in penetrating wounds of muscle and soft tissue contaminated with clostridial spores borne on fragments of soil, clothing, metal, etc. Strict anaerobes are unable to grow at the Eh values found in normal well oxygenated tissues. The basic prerequisite for anaerobic infection is the development of a low Eh following disruption of the blood supply with associated haemorrhage, oedema, hypoxia and necrosis in the damaged tissue; Eh may be particularly low at the surface of particles of foreign material. Clostridial spores germinate and start to grow in the rich nutrients provided by the necrotic tissue; bacterial growth lowers the pH and Eh further. Host defences are compromised, since cells, antibody and antibiotics cannot penetrate the necrotic area, and phagocytosis and intracellular killing are impaired under anaerobic conditions. Pathogenic clostridia survive and are able to exploit this situation, producing a battery of toxins and enzymes that are

thought to contribute to tissue digestion and killing of phagocytes. Increased pressure due to oedema, haemorrhage and gas produced by the bacteria further restricts blood perfusion and helps to spread infection into adjacent healthy tissue. In severe infections systemic illness with prostration, shock and death may rapidly follow; this is conventionally attributed to toxaemia as it may occur without bacterial invasion of the blood, though the toxin(s) involved have not been well characterised (see below).

In civilian practice clostridial wound infection is relatively rare and, although it remains a dramatic and life-threatening disease, the full picture of gas gangrene is seldom seen (see Willis, 1977, chap. 9). The old name persists in common use although gas production is a late and relatively uncommon feature; clostridial myonecrosis describes the disease more accurately. The critical factor in the development of gas gangrene is the anaerobic condition of the wound, which allows contaminating spores to germinate and grow. Clostridial spores are widespread in dust, soil, clothing and skin, and contamination of accidental or operative wounds continues to occur; however, infection is prevented by prompt cleansing with removal of devitalised tissue and restoration of adequate oxygenation. Antibiotics may have played some part in limiting the disease but early effective surgery is much the most important factor in prevention and treatment of gas gangrene. In this connection it should be appreciated that C. perfringens and other anaerobic species are not uncommonly present in the exudate from surgical wounds but that they do not cause infection or delay healing if there is adequate drainage and oxygenation (Gorbach & Thadepalli, 1975; Smith, 1975,

chap. 18); similarly, anaerobic cellulitis is a localised soft-tissue clostridial infection that does not go on to invade muscle tissue or produce gas gangrene (MacLennan, 1962; Finegold, 1977, chap. 13). Gas gangrene in Britain now occurs more usually after elective surgery than following injury; the importance of special precautions in operations on the hip and thigh muscles of the elderly, diabetics or others with relative arterial insufficiency is well recognised (Parker, 1969).

Although the present discussion concentrates on clostridial gas gangrene, a variety of other organisms may be involved in severe necrotising soft-tissue infections (see Finegold, 1977, chap. 13; Dellinger, 1981). The emphasis on the classical clostridial infections may distract attention from anaerobic cocci, fusobacteria and other Gram-negative anaerobes that are increasingly cultured from such conditions. It is salutary to bear in mind that clostridial myonecrosis may have parallels with less well defined infections involving other strict anaerobes, and that some of the features of infection may be attributable generally to the anaerobic condition of the tissues rather than specifically to properties of the invading micro-organism. Not uncommonly, more than one organism can be isolated; it appears that there may be pathogenic synergy between a variety of different anaerobic species. It is also well recognised that facultative anaerobes may help to create conditions appropriate for strict anaerobes by reducing the oxygen levels in the affected tissue, e.g. in Meleney's synergistic gangrene and related conditions (see Finegold, 1977, chap. 13; Willis, 1977, chap. 6). In mixed infections it is difficult to determine which organisms are primary

pathogens and which merely contaminants, able to multiply in the wound but not to cause infection on their own. This situation has been most extensively explored with the clostridia; the pathogenic potential of other strict anaerobes, alone or in combination, is still poorly understood, and progress will depend on further application of the recently developed techniques for reliable culture and identification of these species.

The clostridia responsible for gas gangrene infections were reviewed by MacLennan (1962) and Willis (1969, chap. 9). There is general agreement that the majority of infections can be attributed to C. perfringens type A, C. septicum and C. novyi type A, either alone or in combination. C. perfringens is most commonly implicated (50-80% of cases in various surveys), with the other two competing for second place (c. 20-40%). C. perfringens and C. septicum are comparatively easy to culture and identify; it may be that C. novyi has been overlooked and is relatively under-reported in some surveys. Each of these three species is undoubtedly pathogenic in its own right, and this is supported by their ability to reproduce the infection when pure cultures are inoculated into experimental animals. A number of other clostridial species that are non-pathogenic for laboratory animals may also be isolated from gas gangrene wounds, e.g. C. sporogenes and C. bifermentans; these are assumed to be contaminants and of little significance in the infection. Other clostridia may occasionally contribute to gas gangrene, e.g. C. sordelli, C. histolyticum, C. fallax; these may be pathogenic in experimental infections but are seldom encountered in gas gangrene, and very rarely as the sole isolate.

General considerations of bacterial pathogenicity and virulence determinants were outlined above. Clostridia are primarily commensals in the gut or saprophytes in soil and cause gas gangrene only when introduced into the tissues by injury; mechanisms of adhesion or localisation are not important because the organisms are implanted directly into susceptible tissues. The ability to form spores may be considered of significance for clostridial wound infection since these resistant, dormant forms survive better than vegetative anaerobes in the environment or on skin and this increases their chances of gaining access to injured tissue. Pathogenic clostridia owe their virulence to factors that enable them to evade host defences, grow in the tissues and cause infection. C. perfringens is the only pathogenic clostridial species that produces a capsule; there is evidence that capsules are produced in vivo by virulent strains and that this correlates with their ability to resist phagocytosis and to cause infection (Butler, 1945; Willis, 1969, chap. 2). On the other hand, Keppie & Robertson (1944) found an inverse relationship between the thickness of the capsule and the amounts of toxins produced by strains of C. perfringens, and most studies have found a general correlation between virulence and toxigenicity (see below). Many clostridial toxins are haemolysins or more general cytolysins and it is probable that such products also act against the host's phagocytic cell response at the site of infection. However, such antiphagocytic factors may be of only minor significance in severely damaged necrotic tissues, where inability to penetrate and lack of oxygen may present much more serious problems (Oakley, 1954).

Other areas in which virulence determinants are likely to be of importance in these acute infections are in the initiation of local tissue damage, the extension of the local lesion, and the production of generalised illness and death. The exotoxins and exoenzymes of clostridia have been extensively studied for many years and it is assumed that they play a role in gas gangrene. MacLennan (1962, p. 180) suggested that "the clostridia are primarily and essentially saprophytes, whose toxins are quite as incidental and quite as unimportant to their economy as morphine to the poppy plant or digitalis to the foxglove". Although the ability to cause wound infection is unlikely to be of direct importance in their ecology, it might parallel their ability to digest dead tissue or organic waste in soil. Willis (1969, chap. 9) summarised the classical view of the likely roles of clostridial products, while noting the lack of firm evidence. Thus phospholipase-C (C. perfringens, C. novyi) may damage cell membranes and increase capillary permeability; collagenase (C. perfringens, C. histolyticum) may break down collagen barriers in the tissues; hyaluronidase (C. perfringens) may facilitate spread of organisms through the tissues; and toxic or digestive roles can easily be suggested for other products such as DNA-ase (C. perfringens, C. septicum), lipase (C. novyi) and protease (C. histolyticum) in the local destruction of the tissues. Several clostridia produce toxins that are lethal when injected into experimental animals, e.g. the α-toxins of C. perfringens, C. novyi, C. septicum and C. haemolyticum (all quite distinct toxins); these may contribute to the local damage and it is tempting to attribute the systemic illness and fatal outcome to

toxaemia with these products. Willis (1969, p. 300) cautiously concluded that "while there is much evidence to suggest that the exotoxins produced by clostridia are largely responsible for the nature of the lesion and the systemic manifestations, the modes of action of the toxins and the individual importance of each are complex and uncertain, and not infrequently unknown except in the most general terms".

Toxins and other products of C. perfringens type A

Clostridia produce a variety of exotoxins and other exoproteins; these still play a major part in current schemes of identification along with biochemical characterisation (see Holdeman, Cato & Moore, 1977; Willis, 1977, chap. 4). The extracellular products are identified serologically and by their biological activities, e.g. lethal, dermonecrotic, haemolytic or enzymatic properties. They have traditionally been called toxins and distinguished by different letters of the Greek alphabet; however, it should be appreciated that this was a convention of nomenclature and that their toxic role *in vivo* is often highly speculative. The toxins produced by *C. perfringens* were listed by Willis (1969, chap. 2); all types produce the α-toxin but type-A strains are distinguished from types B-E by the other toxins produced. In addition to α-toxin, type-A strains produce an oxygen-labile haemolysin (θ-toxin), collagenase (K-toxin), hyaluronidase (μ-toxin), DNA-ase (ν-toxin) and neuraminidase; in addition food-poisoning strains produce the enterotoxin. The α-toxin is the major lethal toxin produced by type-A strains,

though strains of types B-E also produce other lethal toxins; these organisms may be associated with a variety of veterinary infections but are not involved in human myonecrotic infections (see table Intro/VI).

Alpha toxin. There is general agreement that the α-toxin is the most significant factor in production of gas gangrene by C. perfringens type A, although it is recognised that the evidence is incomplete (MacLennan, 1962; Willis, 1969, chap. 2; Ispolatovskaya, 1971; Smith, 1979; Stephen & Pietrowski, 1981, chap. 3). The α-toxin was characterised as a lecithinase or phospholipase-C by MacFarlane & Knight (1941), the first bacterial toxin to be shown to have an enzymic action. It also has lethal, dermonecrotic, haemolytic and cytolytic activites; early studies were complicated by difficulties in separating the phospholipase from other toxic or membrane-damaging products of the organism (Wadstrom, 1978) but recent studies with highly purified preparations have confirmed that these different biological activities are associated with a single molecular species (Smyth & Arbuthnott, 1974; Avigad, 1976; Mollby, 1978; Takahashi, Sugahara & Ohsaka, 1981).

The structures of some common phospholipids and the point of action of various phospholipases and sphingomyelinases are shown in fig. Intro/2. The phospholipids found in greatest amounts in cell membranes are phosphatidylcholine (lecithin), phosphatidylethanolamine, phosphatidylserine and sphingomyelin. The C. perfringens α-toxin is a phospholipase-C, hydrolysing phosphatidylcholine to produce soluble phosphorylcholine and insoluble diglyceride; this

Table Intro/VI

Disease conditions produced by different types of Clostridium perfringens

Type	Disease[a]
A	Gas gangrene of man and animals Food poisoning Equine grass sickness Necrotising colitis and enterotoxaemia of horses
B	Lamb dysentery Enterotoxaemia of foals, sheep, goats
C	Enterotoxaemia of sheep (struck), calves, lambs, piglets Necrotic enteritis (Pig-Bel)
D	Enterotoxaemia of sheep, lambs (pulpy kidney), goats, cattle
E	Role in pathogenicity unclear, found in sheep and cattle

[a] From McDonel (1980).

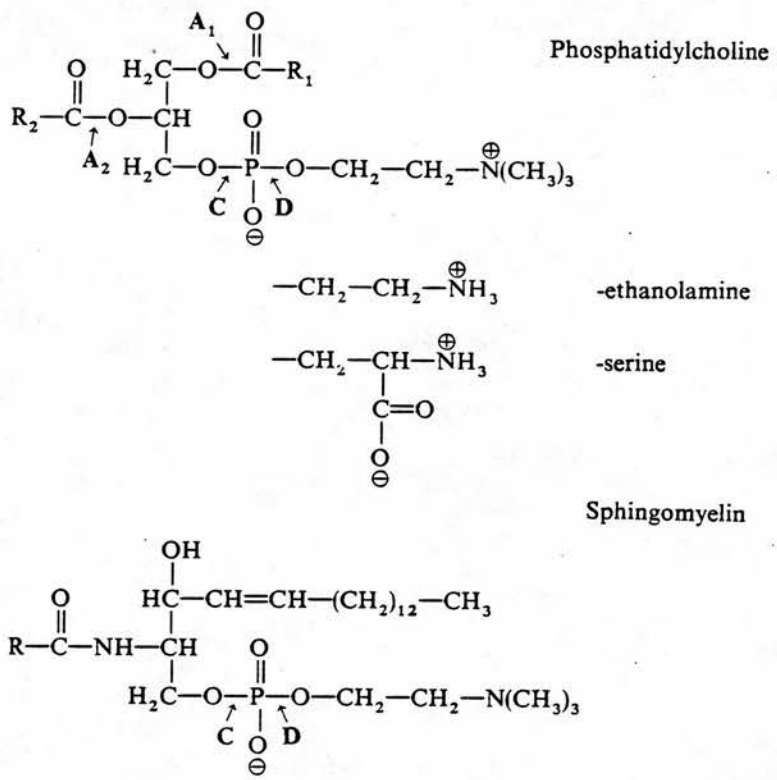


Fig. Intro/2 - Structure of common cell-membrane phospholipids; the position of hydrolysis by phospholipases A_1, A_2, C and D is indicated (from Mollby, 1978).

is the basis of the Nagler reaction used for identification of phospholipase-producing clostridia on serum or egg-yolk agar (Willis, 1977, chap. 3). Although Pastan, Macchia & Katzen (1968) suggested that activity against lecithin and against sphingomyelin was attributable to the presence of two separate enzymes, later studies (Smyth & Arbuthnott, 1974; Mollby, 1978) have not confirmed this and have shown that purified phospholipase-C is active against both substrates. Substrate specificity has been investigated with purified phospholipids; activity is markedly dependent on the physical state of the preparation, e.g. the degree of bilayer formation, and activity may be greater against phospholipids on intact cells or membrane preparations (Mollby, 1978).

The mechanism of membrane damage by C. perfringens phospholipase-C has been intensively investigated (see Avigad, 1976; Freer & Arbuthnott, 1976; Mollby, 1978; McDonel, 1980). Membrane damage may occur without actual red-cell lysis; this is illustrated in the phenomenon of hot-cold lysis, which does occur with purified C. perfringens phospholipase-C although it is not as marked as with a specific sphingomyelinase-C produced by Staphylococcus aureus (see Arbuthnott, 1978b). The enzyme requires Ca^{2+} for activity; it is now thought that its influence is predominantly on the substrate rather than directly on the enzyme itself. Zinc is also required, primarily to stabilise the enzyme and protect it against proteolytic destruction in the culture medium (Sato et al., 1978). The exact mechanism by which enzymic hydrolysis of surface phospholipids destabilises the erythrocyte membrane is still debated, but it is quite distinct from that of the oxygen-labile θ-haemolysin (see below); many of the earlier

studies were invalidated by the use of contaminated preparations. Most studies have used red cell membranes, either in intact cells or as erythrocyte ghosts, but similar effects may occur with membranes of other cell types. The effect of the enzyme depends on many variables, including the relative concentrations of different types of phospholipids in the membrane of the target cell, their relative distributions on the inner and outer surfaces of the membrane and their accessibility to the enzyme. Factors such as these, and differences in substrate specificity for various phospholipids and sphingomyelins, presumably explain differences in haemolytic and toxic activities for phospholipase-C enzymes produced by other bacteria, e.g. C. sordelli, C. bifermentans, C. novyi, Bacillus cereus and Pseudomonas species; the C. perfringens enzyme has a broad substrate range and is much the most toxic (see Mollby, 1978).

The α-toxin can damage a variety of other cells besides red cells. The original studies of the effects on muscle (e.g. Robb-Smith, 1945) were made with impure toxin preparations but many of the observations of the relative effects of different bacterial products have since been confirmed with pure preparations. Aikat and Dible (1956) showed that the α-toxin was the main factor responsible for digestion of muscle, either in vitro or when injected into experimental animals; they demonstrated that neither collagenase nor hyaluronidase was necessary for the action of α-toxin. The membrane-damaging effects of purified C. perfringens phospholipase-C acting on muscle fibres has been confirmed by Strunk, Smith & Blumberg (1967) and Boethius et al. (1973) and has

also been demonstrated with fibroblasts (Mollby, Thelestam & Wadstrom, 1974). Phospholipase-C damages leucocytes and platelets; platelet damage leads to aggregation and thrombus formation which may contribute further to haemostasis and Eh reduction during infection (Mollby, 1978; McDonel, 1980). C. perfringens α-toxin damages endothelial cells and produces increased capillary permeability, conveniently demonstrated by extravasation of dye at the site of intradermal injection in experimental animals (Willis, 1969, chap. 2); release of histamine from mast cells and mediators from damaged platelets may also contribute to the massive oedema that is a striking feature of gas gangrene (McDonel, 1980).

The effects of injection of C. perfringens α-toxin into experimental animals have been reviewed by Willis (1969, chap. 2), Smith (1975, chap. 7) and McDonel (1980). The results depend markedly on the route of injection. Intravenous injection produces haemolysis, destruction of platelets, widespread capillary damage, oedema, shock and death. The toxin quickly disappears from the blood; Ellner (1961) found that it was not taken up by skeletal muscle or brain but appeared largely in the liver and other internal organs and was rapidly metabolised. Conversely, α-toxin injected by the intramuscular route remains bound to muscle; very little appears in the blood and there are no systemic effects unless very large doses are given. There is oedema and necrosis in the injected muscle; the amount of tissue swelling and disruption is increased by the presence of hyaluronidase, though collagenase appears to have little direct effect (Aikat & Dible, 1956). It seems that α-toxin is rapidly adsorbed to phospholipids and lipoproteins in the local lesion and that little is detectable in

the tissue fluids; free toxin has occasionally been demonstrated in the exudate from rapidly deteriorating gas gangrene wounds but only very rarely from the blood in such cases (see Willis, 1969, chap. 2; McDonel, 1980). Haemolysis is a late and uncommon clinical feature in clinical gas gangrene, though it occurs more commonly with post-abortal clostridial uterine infections. Oakley (1954) argued that small amounts of α-toxin absorbed into the bloodstream might produce serious changes in vital organs in the absence of haemolysis, but it seems unlikely that there is significant α-toxin in the blood in the majority of "toxaemic" gas gangrene patients (Willis, 1969, chap. 9).

Theta toxin. Other C. perfringens products may be involved in production of the local lesion in infected muscle but the evidence suggests that they play a subsidiary role to the α-toxin. The θ-toxin has been purified and characterised (Smyth, 1975). It is one of a group of oxygen-labile haemolysins that are now more commonly described as sulfhydryl- or thiol-activated cytolysins (Bernheimer, 1976; Smyth & Duncan, 1978) because they may affect a range of different cell types; since they may produce significant damage in sub-lytic doses they are more accurately described as membrane-damaging toxins (Arbuthnott, 1978b). Related thiol-activated haemolysins are produced by a number of other pathogenic and saprophytic Gram-positive bacterial species, e.g. C. tetani and several other clostridia, Streptococcus pyogenes, Bacillus cereus, Bacillus thuringiensis; there is cross-neutralisation by antisera within the whole group despite their diverse origins. All are heat-labile and thiol-activated proteins that can be neutralised by

cholesterol, which is the natural receptor in cell membranes. Binding of toxin to cholesterol leads to redistribution of the sterol in the membrane and the formation of functional pores permeable to potassium ions, and eventually haemoglobin. These toxins also produce membrane damage with various other types of cell, including leucocytes, and are lethal on intravenous injection into experimental animals (see Bernheimer, 1976; Smyth & Duncan, 1978). However, there is no evidence that much θ-toxin is produced in vivo or that it plays a significant part in the pathogenesis of gas gangrene (Oakley, 1954) and anti-θ-toxin is not protective in experimental infections (Evans, 1943a).

Other toxins. The collagenase, hyaluronidase and DNA-ase of C. perfringens have not been well characterised and their role in muscle damage remains uncertain (see Oakley, 1954; MacLennan, 1962; Willis, 1969, chap. 2; McDonel, 1980). There is no clear correlation between virulence and in-vitro production by different strains (Ispolatovskaya, 1971). MacFarlane & MacLennan (1945) identified the collagenase and suggested that it played a major role in the disintegration of infected muscle, but Evans (1947) was unable to show a protective effect of anti-collagenase in experimental infection. Aikat & Dible (1956) found that α-toxin was predominantly responsible for muscle damage and were unable to detect any additional effect attributable to collagenase; however, their experiments were criticised by MacLennan (1962) who argued for a role for collagenase in destroying the structure of muscle and helping the spread of infection. Hyaluronidase attacks the matrix material of connective tissue and has been shown to increase the muscle damage produced by α-toxin (Robb-Smith, 1945; Aikat &

Dible, 1956); it, too, is generally postulated to play a role in the spread of damage through infected muscle (Willis, 1969, chap. 2). The DNA-ase of C. perfringens is a non-lethal product that does not produce muscle damage but has been suggested to have leucocidal activity (Robb-Smith, 1945). It might contribute to the poor leucocyte response that is typical of serious myonecrotic infection (Butler, 1945) although other factors such as α-toxin and θ-toxin might equally be incriminated (Oakley, 1954; Willis, 1969, chap. 2).

Enterotoxin. The enterotoxin of food-poisoning strains of C. perfringens type A is a sporulation-specific product, perhaps an actual spore-coat component that is overproduced by food-poisoning strains and may become visible as a parasporal semi-crystalline accumulation in the cell (Duncan, King & Frieben, 1973; Frieben & Duncan, 1973; Labbe & Duncan, 1977). The precise mechanism by which it produces secretion of fluid and electrolytes in the small intestine is still to be elucidated; it is clearly different from the cyclic-AMP-mediated stimulatory mechanism of cholera toxin. C. perfringens enterotoxin produces necrosis of the tips of the ileal villi in experimental animals, probably by direct cell-membrane damage to the brush border of the epithelial cells (McDonel, 1979, 1980). Although the enterotoxin can also produce increased capillary permeability on intradermal injection (Hauschild, 1970) and is lethal when large doses are given by intravenous injection (Stark & Duncan, 1971), it is not thought to play a part in the pathogenesis of gas gangrene. It is produced in large amounts only by food-poisoning strains, many of which are not

virulent on intramuscular injection (Hauschild & Thatcher, 1968). Furthermore, C. perfringens in wound infection is present as rapidly growing vegetative cells with insignificant spore formation; this contrasts with the situation in the gut where vegetative cells in food reaching the small intestine rapidly sporulate, releasing enterotoxin.

Other products. A variety of other bacterial products have been reported in culture supernates of C. perfringens strains, e.g. enzymes that destroy various blood-group substances, fibrinolysin, non-αθδ-haemolysins, "bursting factor" (Fredette, Forget & Vinet, 1962) and "circulating factor" (Ganley, Merchant & Bohr, 1955). These were reviewed by Willis (1969, chap. 2); they are in general poorly characterised and of dubious importance in clostridial myonecrosis. The haemagglutinin of C. perfringens type A was mentioned in an earlier section; since it is produced by laboratory strains but not by fresh isolates (Collee, 1961) it seems unlikely to be related to virulence. The possible role of neuraminidase in clostridial myonecrosis is considered in the Discussion section of this report.

Pathogenesis of C. perfringens type A gas gangrene

Although C. perfringens type A may be involved in a number of other types of infection (see Willis, 1969, chap. 9), gas gangrene is the most important and dramatic condition produced and studies on the pathogenicity of the organism have focused on this. Muscle infection can be produced experimentally in a variety of animals;

the guinea pig has been most widely used, e.g. in the classical studies of Evans (1943a and b) and the more recent investigations by Bullen and his colleagues (Bullen & Cushnie, 1962; Bullen, 1970). Experimental myonecrotic infection of the guinea pig thigh muscles reproduces many of the features of clinical gas gangrene, although it has been suggested that the muscle mass may be too small to provide a full parallel with human infection (Oakley, 1954). For obvious reasons, observations on larger animals, e.g. with experimental gunshot wounds in sheep, have been limited (but see Owen-Smith & Matheson, 1968; Boyd, Thomson & Walker, 1972a; Boyd, Walker & Thomson, 1972b).

The importance of the route of administration, the state of the tissues and the presence or absence of pre-formed toxin in the inoculum administered to experimental animals was established in early studies and was reviewed by Willis (1969, chap. 2). Injection of washed cells of C. perfringens into muscle does not produce infection unless there is a nidus of ischaemic or necrotic tissue. Infection may be initiated by inoculation of whole broth culture into healthy muscle; the amount of pre-formed toxin present in the culture is often sufficient to produce local damage and allow establishment of the organism. However, this procedure does not mimic natural infection, when spores are assumed to initiate infection in the absence of exogenous toxin; furthermore, variations in virulence of organisms in such experiments might reflect variations in their production of toxin(s) in vitro that might not parallel their virulence in vivo.

Calcium chloride has been identified as the agent responsible for early tissue necrosis in wounds contaminated with soil, and it

has been used as standard sclerosing agent in many studies. It should be appreciated that it might also act in other ways, e.g. Ca^{2+} might stimulate the activity of C. perfringens phospholipase-C in vivo as well as in vitro. Princewill (1965) showed that Ca^{2+} might also stimulate germination and outgrowth of clostridial spores; however, the use of washed suspensions of log-phase cells as inoculum avoids variations that might be due to factors influencing spore dormancy and activation. An alternative method for initiation of infection is to mix the bacterial inoculum with adrenaline (Evans, Miles & Niven, 1948); the adrenaline gives transient intense vasoconstriction and a local fall in Eh for c. 2 h without apparent residual damage, but it produces greatly increased susceptibility to infection. Clinical gas gangrene has occurred after intramuscular injection of adrenaline in man; the risks of introduction of clostridial spores from skin along with vasoconstricting substances in this way are well established (e.g. see Leading Article, 1968).

When virulent organisms are inoculated with either of these initiating agents, infection is established and rapidly progresses to produce extensive necrosis and digestion of the thigh muscle mass with collapse and death occurring in 24-48 h. Less virulent organisms may be able to cause local infection but this is contained and the animal survives. The clinical features of human clostridial wound infections were reviewed by MacLennan (1962) who noted that gas gangrene developed in only a proportion of wounds contaminated by clostridia, but in these cases the onset of illness was often sudden and its progress very rapid. The course of

infection depends both on the nature of the wound, in particular the degree of tissue damage and Eh reduction that is produced (see above), and on the virulence of the infecting strain. In experimental infection the initial tissue compromise can be standardised, so that virulence of the challenge strain can be assessed by the size of inoculum required to initiate infection. Other measures of virulence in this model are the severity of local infection and the ability to produce the systemic disease and rapid fatal outcome that parallel the toxaemic phase of human infection.

Various approaches have been developed for analysis of virulence factors that may operate at different stages of infection. As previously described, many of the features of the local tissue damage can be reproduced by exposure of muscle to culture filtrates of C. perfringens strains, either in vitro or in vivo; experiments with preparations containing different proportions of various toxins suggested a major role for the α-toxin, with the possibility of some further contribution from collagenase, hyaluronidase and θ-toxin. In a complementary approach, Evans (1943a and b, 1947) studied the protective effect of antisera containing different proportions of various antibodies for guinea pigs challenged with C. perfringens; there was a general correlation between protection and anti-α-toxin titres, but he was unable to show any protective effect attributable to anti-θ-toxin, anti-collagenase or anti-hyaluronidase, either alone or in combination with anti-α-toxin (see also Kameyama, Sato & Murata, 1972).

The protective effects of antitoxic sera are limited, and the guinea pig appears to provide a good parallel with human infection

in this regard too. Antiserum gives passive protection only when administered before or very shortly after experimental challenge. Its effect is primarily to prevent or abort infection rather than to modify the course of established infection; it is ineffective when given therapeutically in an attempt to prevent systemic collapse and the toxaemic phase of the disease (Evans, 1945a; Bullen, 1970). There is evidence that active immunisation of various animals with clostridial toxoids gives similar protection against the development of gas gangrene on later challenge (Willis, 1969, chap. 9; Boyd et al., 1972b). Active immunisation of soldiers has not been practised on a wide scale but clinical experience of the value of antitoxin in preventing gas gangrene in military casualties was evaluated by Oakley (1954) and MacLennan (1962). Standard preparations of antitoxin are polyvalent, containing antibody to a variety of toxins produced by C. perfringens and also by C. septicum and C. novyi; although the potency of C. perfringens anti-α-toxin could be standardised, the content of antibody to other products of the organism might vary. The difficulties of assessing results obtained under wartime conditions are considerable, with different types and degrees of injury and contamination, and the problem of disentangling the effects of surgery, antitoxin and, latterly, antimicrobial therapy. The most satisfactory studies (e.g. MacLennan & MacFarlane, 1944; MacLennan & MacFarlane, 1945) suggested that antitoxin was of some value if administered within c. 6 h of injury; given later, however, it was ineffective in preventing extension of established disease and ultimate death. These conclusions have been borne out in civilian practice; treatment with antitoxin or antibiotics is of

very limited efficacy unless necrotic tissue can be completely removed by surgery and full oxygenation is restored (Willis, 1969, chap. 9; Weinstein & Barza, 1973; Hitchcock, Demello & Haglin, 1975; Darke, King & Slack, 1977; Finegold, 1977, chap. 19; Garrod, Lambert & O'Grady, 1981, chap. 16).

Many studies have sought to correlate pathogenicity and toxigenicity in C. perfringens. Strains isolated from gangrenous wounds generally produce good levels of α-toxin in vitro; yields of other toxins may be much more varied. A number of studies have shown that commensal and environmental strains may produce α-toxin levels as high as or higher than those produced by known pathogenic strains (e.g. Keppie & Robertson, 1944; Price & Shooter, 1964; Mollby et al., 1976). Butler (1943) found that laboratory strains of C. perfringens may lose virulence during subculture and regain it after animal passage; this may not be important in nature since infection is normally with environmental strains. Bullen (1970) drew attention to discrepancies between α-toxin production and virulence for individual strains in a number of published accounts that in general supported a correlation of the two, e.g. Keppie & Robertson (1944), Evans (1945b). Bullen, Wilson & Cordiner (1961) studied two strains of C. perfringens that were of similar virulence, with a minimum lethal dose of c. 10^2 cells, and showed that one produced very much higher levels of various toxins than the other in vitro. Bullen (1970) also emphasised discrepancies between anti-α-toxin titres and protection against different challenge strains in the studies of Evans (1945a) and suggested that factors other than α-toxin were of major significance in the pathogenesis of gas gangrene.

Bullen & Cushnie (1962) studied the growth of a virulent but poorly toxigenic strain of C. perfringens when injected into guinea pig thigh muscles in experiments designed to assess whether invasiveness of the organism might be distinct from toxigenicity. When muscle was damaged by $CaCl_2$ injection, the presence of anti-α-toxin did not reduce the rate of growth of the inoculum in the muscle, and did not prevent extension of the lesion into healthy tissues; this was not due to failure of penetration of antibody as large amounts were demonstrable in the damaged tissues. By contrast, when adrenaline was used as initiating agent passive immunisation was much more effective in halting bacterial growth and protecting the animals. Thus the invasiveness of the organism was not solely due to its toxins, but was also dependent on the state of the host's tissues. Later studies on the efficacy of anti-α-toxin identified other factors that may affect the balance between host and pathogen. Bullen, Cushnie & Rogers (1967) showed that intravenous injection of ferric ammonium citrate abolished the protective effect of antiserum in animals given adrenaline as initiating agent. It is now appreciated that the ability to obtain Fe^{3+} from the tissues is a decisive factor for virulence in a variety of bacterial species, including Pasteurella septica, Escherichia coli and mycobacteria as well as C. perfringens (Rogers, Bullen & Cushnie, 1970; Bullen et al., 1978). Virulent strains produce iron-binding proteins that can successfully compete with the host's transferrin for the small amounts of free Fe^{3+} in the tissues; the balance is complex as the availability of iron is also important for the host's phagocytic cells. It appears that the bacteriostatic effect of partially saturated transferrin is an

important component in the host's defence against challenge with C. perfringens; an increase in available Fe^{3+} removes the limitation on bacterial growth and allows infection to progress despite the presence of antitoxin.

Bullen (1970) argued that the invasiveness of C. perfringens is largely independent of toxin production but depends on the ability of normal muscle tissue to produce bacteriostasis. This is not solely due to the Eh in the tissues, as injection of iron some hours after the effects of adrenaline have worn off can still stimulate bacterial growth again (ferric iron itself does not lower the Eh as it remains in the oxidised form). However, the bacteriostatic effect of serum at normal Eh values (c. +200 mV) is much reduced if the Eh falls to zero or negative values (Bullen, Dobson & Wilson, 1964). C. perfringens is not a very strict anaerobe as it can grow at an Eh value as high as +60 mV and can remove oxygen from its environment; in severe clostridial infection the Eh falls to -400 mV (Bullen, Cushnie & Stoner, 1966). Bullen (1970) proposed that extension of the gangrenous lesion is due to bacterial removal of oxygen from the neighbouring healthy tissue at the edge of the lesion so that Eh falls and the normal bacteriostatic mechanisms are compromised.

Hyperbaric oxygen has been advocated for treatment of gas gangrene (Brummelkamp, 1965; Gottlieb, 1971; Darke et al., 1977; Willis, 1977, chap. 9); opinions vary on its place in modern therapy but there is no doubt that oxygen administration can arrest the progress of the local lesion and gain time before surgical excision proceeds. It was initially suggested that the effect

depends upon inhibition of toxin production or of clostridial growth, but it is also possible that increased oxygen supply to the extending edge of the lesion may act to balance oxygen removal by the bacteria and restore normal bacteriostasis (Bullen et al., 1978).

There is little agreement about the mechanisms that produce the toxaemic phase and death in gas gangrene (e.g. see Oakley, 1954; MacLennan, 1962; Willis, 1969, chap. 9; Bullen, 1970; Smith, 1979; Stephen & Pietrowski, 1981, chap. 3). The failure to demonstrate α-toxin in the blood and the relative rarity of haemolysis in fatal clinical infections (mentioned above) throw considerable doubt on early suggestions that death is due to the lethal effect of circulating α-toxin. The limited protective effect of antitoxin in established disease in animals or man adds to the evidence that α-toxin is not the major factor. MacFarlane & MacLennan (1945) suggested that the toxaemia was due not to α-toxin but to some other toxic substance produced by its action on muscle; it is not due simply to release of products from anoxic muscle as it is not seen with sterile necrotic lesions or other muscle infections (see Oakley, 1954). Production of massive oedema and exudate may be the mechanism at least partly responsible for systemic collapse and the development of shock. Local effects of clostridial toxins on muscle cells and on capillary permeability may be involved, though the failure of antitoxins to prevent death unless necrotic tissue is removed surgically suggests that none plays a major part. Experiments with fatal C. perfringens peritonitis in passively immunised guinea pigs (Bullen & Cushnie, 1963; Bullen et al., 1966) indicated that shock and death followed

a massive loss of fluid and protein into the peritoneum. This was not attributable to recognised toxins or mediators such as histamine and it was ascribed directly to the removal of oxygen by the metabolic activity of the organism; however, the differences between experimental peritonitis and muscle infection should be borne in mind.

It is apparent that the pathogenesis of C. perfringens gas gangrene remains incompletely understood. It seems reasonable to postulate an important role for α-toxin in the initial stages of infection and in the local tissue damage, and it is probable that anti-α-toxin has some protective effect at this stage; however, the role of α-toxin in extension of established infection is much more debatable and there is little evidence to support the original concept of toxaemia with α-toxin as the mechanism leading to death. Most authorities conclude that α-toxin plays an important part, but views range from "It is difficult to escape the conclusion that the syndrome of fatal human or experimental gas gangrene caused by C. perfringens is the integrated sum of all the deranged metabolic events initiated by α-toxin" (Stephen & Pietrowski, 1981, p. 45) to "There is little evidence to suggest that α-toxin, or any other toxin, has any essential role in the pathogenesis of this disease" (Bullen, 1970, p. 274).

It seems that the recognised toxic products of C. perfringens do not account completely for the pathogenicity of the organism. The studies discussed earlier in this Introductory Review suggest a variety of mechanisms, direct and indirect, by which neuraminidase produced by C. perfringens might contribute either to the local tissue damage or to systemic derangements and collapse; such a

role has been suggested on a number of occasions (e.g. Collee, 1962, 1965b; Muller, 1970a, 1974a) although few studies have been designed to test the possibility. Antisera prepared against C. perfringens products have been assessed primarily on the basis of their content of various antitoxins but they may also contain anti-neuraminidase (Warren & Spearing, 1963); the extent to which this component might contribute to their protective effects is not known. The present studies were undertaken in order to clarify the roles that neuraminidase and anti-neuraminidase might play in the production and development of experimental clostridial myonecrosis.

MATERIALS AND METHODS

Bacterial strains

Source and maintenance of cultures. All strains were held in the collection of Prof. J.G. Collee, Microbial Pathogenicity Research Laboratory, Department of Bacteriology, University of Edinburgh. The source of strains that were recently obtained from the National Collection of Type Cultures, Colindale Avenue, London NW9 5HT is shown as NCTC. Other strains were originally obtained from the NCTC and have been held in Prof. Collee's collection for many years; their source is indicated as Prof. Collee and the NCTC numbers are quoted. The remainder of the strains, including some NCTC strains and clinical isolates from a variety of infections, were obtained from various sources at various times and have been held here, sometimes for many years and with many subcultures; the donors of the strains are cited. All strains were maintained as lyophilised stock with periodic subculture in cooked-meat broth (CMB) and relyophilisation. For the present studies all strains were preshly grown in CMB from lyophilised stock and their purity and identity were carefully checked in microscopic, cultural and biochemical studies.

Strains of Clostridium perfringens (C. welchi). The standard strain used in these studies (L2Ab) was a laboratory sub-strain derived from a classical haemolytic type-A strain obtained from the late Prof. C.L. Oakley, School of Medicine, University of Leeds. Other classical type-A strains were: strain L3A, also from Prof.

Oakley; NCTC8237, from the NCTC; and strains C1 and 032, isolated from wound infections in Edinburgh by Prof. Collee. All strains were characterised biochemically by the tests listed by Willis (1977, p.113) and their identity was confirmed by production of opalescence on half-antitoxin egg-yolk agar (EYA) plates that was inhibited by C. perfringens type A antitoxin (see below). In addition, strain L2Ab was typed by toxin-antitoxin neutralisation tests in a guinea pig (Cruickshank et al., 1975, p.475). Strains CW6 and CW7 were strains of C. perfringens isolated from human wound infections and identified biochemically in this laboratory but found not to produce phospholipase-C (α-toxin); they were submitted to Dr A.T. Willis, Central Public Health Laboratory, Colindale, London, who confirmed their identity. Strain NCTC11144 is a phospholipase-negative strain obtained from the NCTC.

Typical non-haemolytic, heat-resistant, food-poisoning type-A strains of Hobbs' types 1-4 (Nos 8359, 8238, 8239 and 8247 respectively) were obtained from the NCTC; strains of Hobbs' types 5-24 were provided by Dr Betty Hobbs, Central Public Health Laboratory, Colindale, London. Strains 029 and 153 were typical food-poisoning strains isolated from separate food-poisoning outbreaks in Edinburgh (Collee, 1965b); they were submitted for serotyping to Dr Hobbs who reported that they were both type-13 strains. Strains 611 and 4621 were provided by Dr Hobbs: these were non-haemolytic type-13 strains but were not responsible for actual food-poisoning outbreaks; strain 611 was isolated post mortem from a patient with colitis and strain 4621 from a patient who died during a food-poisoning outbreak attributed to a different strain of C. perfringens. Reference food-poisoning strains of C. perfringens type

A that do not produce heat-resistant spores (types i-iii, v-xviii) were provided by Dr Hobbs and are referred to as heat-sensitive strains. The identity of all the food-poisoning strains of C. perfringens type A was confirmed by culture on half-antitoxin EYA plates. The haemolytic reactions were tested on horse-blood agar (HBA) as detailed below, but the heat-resistance of the spores was not retested in this laboratory.

A collection of strains of C. perfringens type A that were reported to have altered neuraminidase production (Rood & Wilkinson, 1975) were obtained as follows: strain CN3870 (wild-type) and the mutant strains CM156, CM165, CM198, CM206 and CM209 from Dr R.G. Wilkinson, School of Microbiology, University of Melbourne, Parkville, Victoria 3052, Australia; strains CM173 and CM218 were from Dr J.I. Rood, Food Research Institute, 1925 Willow Drive, University of Wisconsin, Madison 53706, USA. The characters of these strains are reported in Section IIIc and table III/XIV.

Strains of C. perfringens types B-E were as follows: NCTC3110 (type B), NCTC3180 (type C) and NCTC8084 (type E) were from Prof. Collee's collection; strain L8 (type D) was originally obtained from Prof. Oakley. All were identified biochemically and by culture on half-antitoxin EYA plates. In addition, strains NCTC3180 and L8 were retyped by toxin-antitoxin neutralisation tests in guinea pigs.

Strains of other clostridial species. The following strains were obtained directly from the NCTC: C. chauvoei NCTC8070; C. tertium NCTC541; C. sordelli strains 1340, 2914, 6800, 6801, 6927, 6929 and 8780; C. bifermentans strains 1341 and 6928;

C. novyi (C. oedematiens) strains 9746 and 9692; C. tetani strains 279, 5405 and 9569; C. botulinum strains 7272, 7273, 3732 and 8266; C. difficile NCTC11223; C. sphenoides NCTC507; C. fallax NCTC8380; and C. tetanomorphum NCTC2909.

C. tertium strain CT1, C. sordelli CB4 and C. subterminale CS1 were obtained from Dr B. Watt, Bacteriology Laboratory, City Hospital, Edinburgh. C. absonum strains HA7103, HA7107 and HA9103, and C. paraperfringens 2227, 3-3, G and 9 were from Prof. S. Nishida, Department of Bacteriology, School of Medicine, Kanazawa University, Japan. C. sordelli strains 1734 and P3, and C. bifermentans B4 and 1617 were from Prof. Oakley. C. difficile strains N3 and N6 were from Dr S. Hafiz, University of Sheffield Medical School. C. sporogenes strains 23, 24, 26 and 28 were from Dr Nancy Hayward, Monash University Medical School, Australia.

The remaining strains of clostridia were from Prof. Collee's collection: C. chauvoei CC2; C. sordelli CB2 and CB3; C. bifermentans NCTC506; C. tetani NCTC540, NCTC5404 and NCTC5413; C. difficile MPRL2, MPRL105 and MPRL174 (recent isolates from the stools of patients with diarrhoea); C. histolyticum NCTC503, NCTC7123, NCTC7124 and CH2; and C. butyricum NCTC7423. C. septicum strains NCTC547, 688/52, 1376/53, 2029/53 and CN3204 were described by Gadalla & Collee (1967). C. novyi strains NCTC538 (GR1A), NCTC6737 (GR3A), GR2A, GR4A, GR1B, NCTC9747 (GR1C), NCTC8145 (GR2D) and NCTC8350 (GR1D) were described by Rutter & Collee (1969).

Unless otherwise stated, all strains were identified to species level by the biochemical criteria given by Willis (1977, p.113). C. tetanomorphum NCTC2909 is thus named in the NCTC

catalogue but has been reclassified as C. cochlearium (Holdeman et al., 1977, p. 97; Nakamura et al., 1979). The biochemical reactions of C. butyricum NCTC7423 were confirmed to be as given by Willis (1969, p.14). C. subterminale CS1 was identified by the biochemical criteria, including gas-liquid chromatography (GLC), of Holdeman et al. (1977, p.80); this strain was also submitted to Dr Willis who confirmed its identification. The identity of the strains of C. septicum and C. chauvoei was further confirmed with immunofluorescent diagnostic antisera (anti-C. septicum, code FC07; anti-C. chauvoei, code FC04: Wellcome Diagnostic Reagents Ltd, 303 Hither Green Lane, London SE13 6TL). The strains of C. sordelli and C. bifermentans were also tested on half-antitoxin EYA plates as for C. perfringens; all strains produced opalescence that was inhibited by the antitoxin (Willis, 1977, p.82). The biochemical distinction between C. sordelli and C. bifermentans was further investigated by tests for fermentation of mannose and sorbitol, and for growth inhibition by mannose (see table IV/IV). The identity of C. tetani strains was confirmed by neutralisation tests in mice (Willis, 1977, p.116). C. difficile strains were further characterised by production of typical colonies on CCFA medium (George et al., 1979) and by GLC (Holdeman et al., 1977, p.80). C. botulinum strains were not retested for biochemical reactions but their identity and type was confirmed with immunofluorescent diagnostic antisera (anti-type A, code FC01; anti-type C, code FC02; anti-type E, code FC03: Wellcome Diagnostic Reagents Ltd). The strains of C. absonum and C. paraperfringens were not reidentified biochemically but their reactions were checked on

half-antitoxin EYA plates; C. absonum strains produced a broad zone of opalescence that was partially inhibited by the C. perfringens antitoxin and C. paraperfringens strains produced a narrow zone that was completely inhibited by the antitoxin (Nakamura et al., 1973). Cato, Holdeman & Moore (1982) have suggested that C. paraperfringens strains are identical with C. barati and should be reclassified under the latter name.

Strains of Bacteroidaceae. Many of the strains were characterised in this laboratory and described by Duerden et al. (1976) or Deacon, Duerden & Holbrook (1978). The GNAB and WPH strains were isolated from various sites in healthy adults in this laboratory, or from clinical material by colleagues in the diagnostic laboratories in the Bacteriology Departments of the Royal Infirmary and the Western General Hospital, Edinburgh. The other strains studied were as follows: Bacteroides eggerthi NCTC11155, B splanchnicus NCTC10825 and NCTC10826, B. asaccharolyticus NCTC9337 and Fusobacterium varium NCTC10560; these were all obtained directly from the NCTC. B. uniformis VPI11227, B. variabilis VPI11368 and B. melaninogenicus ss. levi VPI3300 were obtained from Dr Lillian V. Holdeman, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24060, USA. B. oralis VPI9958, B. oralis (B. buccalis) VPI8906D, B. bivius VPI5540, VPI6318, VPI6822 and VPI7880, and B. disiens VPI7852 and VPI8057 were provided by Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich NOR 70F. B. ruminicola strains B38024, B38080 and B56029 were provided by Dr T. Mitsuoka, Animal Pathology Laboratory, Institute of Physical and

Chemical Research, Wako, Saitama 351, Japan. B. corrodens strains 143A and 151RV were provided by Dr A.L. James, Department of Chemistry, Faculty of Science and Technology, Newcastle upon Tyne Polytechnic.

The classification of the Bacteroidaceae has been much altered and refined over the past decade; current schemes are based mainly on the biochemical activities of the organisms (Holdeman et al., 1977). The strains used in the present study were all identified to species level by the identification scheme developed in this laboratory (Duerden et al., 1976, 1980), including GLC as described by Deacon et al. (1978). A number of the strains have been reclassified in recent years. The former subspecies of B. fragilis are now given separate species status. B. thetaiotaomicron is further subdivided into the species B. thetaiotaomicron, B. eggerthi and B. uniformis; Werner (1974) demonstrated that sugar fermentation tests discriminated reliably between the various species in this group and further distinguished B. variabilis from B. uniformis by differences in fermentation of rhamnose and trehalose. Strain ATCC8492, originally the reference strain of B. thetaiotaomicron, is now classified as B uniformis. The classification of the B. melaninogenicus - B. oralis group of strains has also been considerably revised, with less emphasis placed on pigment production as the criterion for classifying strains as B. melaninogenicus. Because of the basic difference in sugar utilisation, B. melaninogenicus ss. asaccharolyticus has been given separate species status as B. asaccharolyticus (Finegold & Barnes, 1977) while B. melaninogenicus is now subdivided into the subspecies melaninogenicus, levi and intermedius. Strain ATCC15930

was originally a reference strain of B. oralis but has been reclassified as B. melaninogenicus ss. melaninogenicus (Holbrook & Duerden, 1974). Strain NP333, originally described as B. oralis, was provisionally reclassified as B. ruminicola on the basis of xylose fermentation (Duerden et al., 1980) but Shah & Collins (1981) re-examined a range of former B. oralis strains and reallocated strain NP333 to the new species B. pentosaceus; in the same study they reallocated B. oralis VPI8906D to the new species B buccalis. Strain WPH15, previously B. asaccharolyticus, is now reclassified as B. gingivalis by the GLC criteria of Kaczmarek & Coykendall (1980; Coykendall, Kaczmarek & Slots, 1980). B. corrodens strains 143A and 151RV were confirmed to be strictly anaerobic and urease-positive; Jackson & Goodman (1978) have suggested that such strains should be renamed B. ureolyticus to distinguish them clearly from the CO_2-dependent urease-negative strains now classified as Eikenella corrodens. The strains previously classified as B. ochraceus are not strictly anaerobic but require CO_2 for growth; they are now classified in a separate genus, Capnocytophaga (Newman et al., 1979; Williams, Hollis & Holdeman, 1979).

Culture Media

Nutrient broth (NB) was prepared according to the manufacturer's recommendations (Nutrient Broth No. 2, Oxoid Ltd, Wade Road, Basingstoke, Hampshire RG24 0PW).

Cooked-meat broth (CMB) was prepared as described by Cruickshank et al. (1975, p.122), but with Nutrient Broth No. 2 (Oxoid Ltd) replacing the peptone infusion broth.

Todd-Hewitt broth (THB) was prepared according to the manufacturer's recommendations (Oxoid Ltd). In early experiments the pH was adjusted to 7.4 before autoclaving but it was found that the pH of THB sometimes rose considerably on storage. In later experiments the pH was adjusted after autoclaving, by the addition of membrane-filtered 1.0M HCl to the sterile broth before use.

Proteose peptone water (PPW5) contained (g/litre): Proteose Peptone (Difco Laboratories, PO Box 14B, Central Avenue, West Molesey, Surrey KT8 0SE; code 0120) 50 g and NaCl 5 g. It was adjusted to pH 7.0 and was clarified by filtering through Whatman No. 1 paper before autoclaving at 121°C for 15 min. To improve the growth of strains of C. novyi, L-cysteine (Koch Light Laboratories Ltd, Poyle Estate, Willow Road, Colnbrook, Slough, Berkshire SL3 0BZ) and dithiothreitol (BDH Chemicals Ltd, Poole, Dorset BH12 4NN) were added (Moore, 1968); the supplemented broth is referred to as PPW5S. A freshly prepared concentrated (x 50) solution containing cysteine and dithiothreitol was passed through a membrane filter (0.2-μm pore) and added aseptically to the steamed medium just before inoculation with the test organism. The final concentrations were: cysteine, 1 mg/ml; dithiothreitol, 90 μg/ml.

Supplements for broth cultures of Bacteroides strains. CMB was used without added growth factors, but all other broth media used for culture of Bacteroides strains were supplemented with

haemin (BDH Chemicals Ltd) and menadione (Sigma London Chemical Co. Ltd, Fancy Road, Poole, Dorset BH17 7NH) as a routine. Membrane-filtered solutions were added aseptically to the steamed media just before inoculation as described by Deacon et al. (1978). The final concentrations were: haemin, 5 µg/ml; menadione, 1 µg/ml.

Thioglycollate broth (TGB) was prepared according to the manufacturer's recommendations (Thioglycollate Medium, fluid, without dextrose or Eh indicator, code 11727; BBL, supplied by Becton, Dickinson UK Ltd, York House, Empire Way, Wembley, Middlesex HA9 OPS) with the addition of yeast extract (Oxoid Ltd) and sodium succinate (BDH Chemicals Ltd). The final concentrations were: yeast extract, 0.25% (w/v); sodium succinate, 0.25% (w/v).

Digest broth (DB) was prepared as described by Cruickshank et al. (1975, p.111) but with best beef steak in place of horse flesh; the $NaHCO_3$ was omitted and the final pH was adjusted with NaOH to 7.4-7.6 before sterilisation by autoclaving.

PPY broth medium was prepared as described by Deacon et al. (1978).

BM broth medium was prepared as described by Deacon et al. (1978) but 0.25% (w/v) sodium succinate was added and horse serum was omitted.

Trypticase glycogen medium (TGM). The broth medium for production of phospholipase-C by C. perfringens contained: Trypticase Peptone (BBL), 15 g; $Na_2HPO_4.12H_20$, 5.6 g; KH_2PO_4, 0.5 g; and $MgSO_4.7H_20$, 0.2 g. These were dissolved in 1 litre

distilled water and autoclaved at 121°C for 20 min; the pH after autoclaving was 7.1. To this was added 50 ml of autoclaved 20% (w/v) glycogen solution (BDH Chemicals Ltd; 38042).

Horse-blood agar (HBA) was used for characterisation of the haemolytic patterns of food-poisoning strains of C. perfringens; it contained Columbia Agar Base (Oxoid Ltd) with 10% (v/v) Defibrinated Horse Blood (Oxoid Ltd). For routine cultures and purity checks, blood agar (BA) containing 5% (v/v) outdated human blood was used.

Egg-yolk agar (EYA) was prepared as described by Cruickshank et al. (1975, p.186) using 5% (v/v) Egg-yolk Emulsion (Oxoid Ltd) in Columbia Agar Base (Oxoid Ltd).

Bacterial Culture Studies

Method of anaerobic culture. BTL jars (Baird & Tatlock Ltd, Chadwell Heath, Essex) were used with room-temperature-active catalyst sachets supplied by BTL. Jars were filled with hydrogen and 10% (v/v) CO_2 according to the standardised procedure described by Collee, Rutter & Watt (1971). Broth media were held in a steamer at 100°C for 30 min (pre-steamed) and promptly cooled to 37°C just before inoculation. The caps of bottles were loosened when they were put into anaerobic jars. Unless otherwise stated, all cultures were incubated at 37°C.

Strains were held as lyophilised stock with periodic subculture in CMB and relyophilisation. For the present studies, all strains were freshly grown in tubes (10 ml) of CMB and their

purity and identity were carefully checked in cultural and biochemical studies. In general, subcultures were made in tubes (10 ml) of the test culture medium. The initial inoculum was derived from a culture in CMB incubated anaerobically at 37°C for 18 h; the broth was shaken to resuspend the organisms and the cooked-meat particles were allowed to settle before the inoculum was taken.

Observation of haemolytic effect with strains of C. perfringens. Strains were grown anaerobically on HBA plates for 18 h at 37°C with 10% CO_2. Strains graded as haemolytic produced clear haemolysis, with or without a surrounding zone of incomplete haemolysis; non-haemolytic strains produced no effect, or only a zone of faint, incomplete haemolysis (see Section IIId).

Biochemical characterisation of clostridia. Fermentation reactions were tested in tubes (10 ml) of CMB with the addition of 1% (w/v) of the appropriate substrate (glucose, maltose, lactose, sucrose and, for C. bifermentans/C. sordelli strains, mannose and sorbitol). The inoculum was c. 0.02 ml of a 24-h CMB culture of the test organism. Cultures containing the appropriate substrates were incubated anaerobically at 37°C for 24 h along with a control culture in CMB with no added substrate. The pH of the cultures was measured with a pH meter and the value obtained with each test substrate was compared with that in the substrate-free control. A fall in pH of >0.5 units was accepted as a positive fermentation test result (see Rutter, 1970). For detection of urease production, similar tests were performed in CMB containing 1% (w/v) urea; a final pH of >8.0 was regarded as positive. Indole

production was detected with xylene extraction of the CMB culture before addition of Ehrlich's reagent (Holdeman et al., 1977, p.127). Gelatinase activity was demonstrated with gelatin charcoal disks (Cruickshank et al., 1975, p.178) in CMB cultures. For differentiation of C. sordelli and C. bifermentans, tests for growth inhibition by mannose were performed as described by Nakamura et al. (1975) with 24-h cultures in PPY broth containing 1% (w/v) mannose.

Plate tests for phospholipase-C production by clostridia. Strains were cultured anaerobically for 18 h at 37°C on EYA plates. Strains that produced a broad zone of opalescence in the medium surrounding the area of growth were recorded as phospholipase-positive. A few strains that produced only a small zone of opacification beneath the area of the colonies were regarded as weak phospholipase producers. Phospholipase-negative strains produced no effect in the EYA medium. The phospholipase produced by various clostridia was further characterised by observing inhibition of phospholipase action on half-antitoxin EYA plates (Cruickshank et al., 1975, p.474) with C. perfringens type A antitoxin (Clostridial Diagnostic Serum, code CD01; Wellcome Diagnostic Reagents Ltd).

Bacterial counts. Counting methods were as described by Cruickshank et al (1975, pp.306-307). Samples were diluted as appropriate in 10- or 100-fold steps in NB medium that had been pre-reduced by steaming and promptly cooling to 37°C just before use. Total counts were made microscopically in a cell-counting chamber of 0.1 mm depth with Thoma ruling (Gelman Hawksley Ltd, 10 Harrowden Rd, Brackmills, Northampton NN4 OEB). Separate counts

were made of total bacteria and of colony-forming units (cfu); in practice these were very similar as few bacteria were in clumps of 2 or more units even in early log-phase cultures. Viable counts were performed in triplicate with 0.02-ml samples spread with disposable glass spreaders over BA plates and incubated anaerobically at 37°C for 18 h. Spore counts were made using 0.5-ml samples that had been heated in glass tubes (75 x 13 mm) in a waterbath at 70°C for 20 min and rapidly cooled before performing the viable count.

C. perfringens growth-curve experiment. A fresh batch of PPW5 broth (600 ml) was prepared in a conical glass flask, a magnetic stirrer bar (PTFE-covered) was added, and a fine-bore (0.75 mm) PVC sampling tube was passed from the bottom of the flask through the cotton-wool bung. This apparatus was autoclaved at 121°C for 15 min. The flask was then put in an anaerobic jar with the sampling tube passing through the side-arm; an air-tight seal was made at the side-arm with a rubber bung. The sampling tube was clamped, the lid of the jar was sealed and an anaerobic environment was produced with a Gaspak H_2 and CO_2 generator envelope (BBL, supplied by Becton, Dickinson UK Ltd). The jar was placed on a magnetic stirrer (200-250 rpm) in an incubator and the sampling tube was led to the outside of the incubator so that the door did not have to be opened thereafter; the temperature of the incubator was maintained at 37.0-37.2°C throughout the experiment. The use of the Gaspak system produced a positive pressure in the culture flask that allowed samples to be taken through the tube into a syringe at intervals. The dead space in the tubing was <0.5 ml,

and the first 2 ml of fluid withdrawn was discarded in order to wash out the tube before each sample was taken.

After holding the culture medium uninoculated for a preliminary period of 18 h at 37°C, an inoculum of C. perfringens strain L2Ab was introduced through the sampling tube with precautions to ensure that all of the inoculum was washed through into the culture flask. The inoculum contained 4 ml of a 10^{-5} dilution of a 24-h anaerobic culture in CMB medium; this contained c. 1.5×10^4 organisms (total count) and was calculated to produce an initial count of c. 20 organisms/ml in the bulk PPW5 culture. At intervals after inoculation, 10-ml samples of culture were withdrawn and used immediately for bacterial counts; they were then cooled quickly to 4°C and 5-ml samples of supernate (800 g for 1 h at 4°C) were stored at -20°C until assayed for neuraminidase. Intermediate samples of 2-ml volume were used for bacterial counts when it was not desired also to test for neuraminidase. A sample of broth taken just before inoculation of the culture was subcultured aerobically and anaerobically on BA plates for 24 h at 37°C to confirm that the original medium was sterile. Thereafter, purity checks were made in the same way at 12-h intervals.

Stock neuraminidase preparations from bulk culture supernate of C. perfringens strain L2Ab in PPW5 broth (P9). A bottle containing 100 ml of pre-steamed PPW5 broth was seeded with 1.0 ml of an overnight CMB culture of strain L2Ab and incubated anaerobically at 37°C for 48 h; 20 ml of this starter culture was then used as inoculum for each of three 600-ml narrow-mouthed Winchester bottles

containing 500 ml pre-steamed PPW5 broth. After anaerobic incubation at 37°C for 48 h, the cultures were centrifuged (1700 g for 1 h at 4°C) and the culture supernates were pooled and stored at 4°C. The pooled supernate was passed through a Seitz filter and the filtered supernate (P9) was stored at -20°C for some months.

A sample (82 ml) of P9 material was thawed and, after centrifugation (2400 g for 30 min at 4°C) to remove a slight precipitate, the supernate was dialysed at 4°C in 25-mm Visking Cellophane tubing for 72 h against running tap water, and then against a large excess of distilled water for 24 h; the final volume after dialysis was 100 ml. This dialysed preparation (P9D2) had strong neuraminidase activity and was stored in 5-ml "bijou" bottles at -20°C. No phospholipase-C (α-toxin) activity was demonstrable in tube tests when incubated with lecithovitellin substrate at 37°C for 1 h (Holding & Collee, 1971). Two further dialysed samples were similarly prepared and membrane-filtered before storing at -20°C. Preparation P9D3 (final volume 218 ml) was made from 200 ml of P9, and P9D4 (385 ml) from 345 ml of P9. For the neuraminidase activity in these stock enzyme preparations, see Section IIf.

Serial culture of clostridial strains for detection of neuraminidase production. In standard tests with strains of C. perfringens and other clostridial species, the supernate of the second serial 48-h culture in the test medium was used. An inoculum (0.1 ml) of the CMB culture was added to 10 ml of pre-steamed test medium and incubated anaerobically at 37°C for 48 h. The second serial culture was made similarly, with an inoculum (0.1 ml) from this culture. When desired, further serial cultures were made in

the same manner. All strains of C. perfringens grew well in the test media but when strains of other clostridial species did not grow, or grew very poorly, in a particular medium, a further attempt was made with a larger inoculum (1.0 ml) and incubating each serial culture for 96 h.

Growth was estimated visually and was recorded as follows: +++, dense turbidity; ++, moderate turbidity; +, light turbidity, or faint turbidity with many cells seen by microscopy in a wet film; ±, faint turbidity with few cells present on microscopy; -, no turbidity, or no cells seen on microscopy.

The purity of growth in each culture was carefully checked by subculture aerobically and anaerobically for 18-24 h at 37°C on BA plates. When the culture supernate was to be tested for neuraminidase activity, cultures were centrifuged (800 g for 1 h at 4°C) and 5-ml samples were stored at -20°C until the assay. In addition, the centrifuged supernates of C. botulinum cultures were membrane-filtered (0.2 μm pore) to ensure removal of all organisms.

Serial culture of C. perfringens strains for detection of cell-associated neuraminidase. For the experiment presented in table III/X, five serial anaerobic cultures of C. perfringens strains L2Ab and Hobbs' type 2 were made in PPW5 broth, incubated on each occasion for 48 h at 39°C. Cultures 1, 2 and 3 were made by transferring 0.1 ml into 10 ml medium as for standard serial cultures. For the fourth culture a 1.0 ml inoculum was added to 100 ml medium and, for the fifth, 10 ml was added to 500 ml. Samples (5 ml) of centrifuged supernates (800 g for 1 h at 4°C) from cultures 1-4 were stored at -20°C. After centrifugation

(2000 g for 1 h at 4°C), the supernate of the fifth serial culture was stored at -20°C and the packed cells were resuspended in 20 ml sterile normal saline. The cells were washed three times in saline (2000 g for 1 h at 4°C) and the washed cells were resuspended in 20 ml saline. The resuspended washed cells and samples of supernate from each washing were held at -20°C.

Samples (100 ml) of the supernate of the fifth serial cultures were concentrated in 2.5-cm Visking Cellophane tubing exposed to polyethyleneglycol MW 6000 for 20 h at 4°C, and thereafter dialysed against large quantities of distilled water for 72 h at 4°C.

For preparation of cell extracts, the washed cell suspension was thawed and four 3-ml samples of each organism were subjected to ultrasonic disruption (Ultrasonic Disintegrator; MSE Scientific Instruments, Manor Royal, Crawley, Sussex RH10 2QQ) for 1 h with the samples held at 0-1°C in an ice bath. This produced a great decrease in turbidity in the samples, and phase-contrast microscopy confirmed a reduction in the number of intact cells to <1% of the original. The treated samples were pooled and the centrifuged supernate (20,000 g for 1 h at 4°C) was stored at -20°C.

Stock preparation of cell extract of C. perfringens strain L2Ab in PPW5 broth (P10). An inoculum (0.1 ml) of overnight CMB culture of strain L2Ab was added to 10 ml PPW5 broth and incubated anaerobically at 37°C for 48 h; 1 ml of this culture was then used as inoculum for 100 ml pre-steamed PPW5 broth. After anaerobic incubation at 37°C for 48 h the culture was centrifuged (12,000 g for 10 min at 4°C) and the packed cells were resuspended in 20 ml

sterile saline; the cells were washed three times in saline (12,000 g for 10 min at 4°C) and resuspended in 20 ml saline. The resuspended cells were subjected to ultrasonic disintegration at 0-1°C for 30 min in 3-ml volumes and the pooled cell extract was stored, uncentrifuged, at -20°C.

Preparation of cell extracts of clostridial species. Cell extracts were prepared from the centrifuged cell deposits of the second serial cultures (10-ml volumes) of a number of strains of clostridial species grown in PPW5 or CMB medium. Cooked-meat particles were allowed to settle before the broth was removed from CMB cultures. All the supernate was removed from the packed cell deposit after centrifugation (1200 g for 90 min at 4°C) and the cells were resuspended in 2 ml sterile normal saline. The resuspended cells were subjected to ultrasonic disintegration for 15-30 min at 0-1°C until the sample was no longer turbid. These uncentrifuged cell extracts were stored at -20°C until assayed for neuraminidase.

Culture of Bacteroides strains in broth media. For standard tests of neuraminidase production by Bacteroides strains, an inoculum (0.2 ml) of overnight CMB culture was added to 10 ml of pre-steamed medium and incubated for 48 h at 37°C. Growth was estimated visually and recorded as +++, ++, +, ± or - as for cultures of clostridial species (see above). Incubation was continued to 96 h to improve growth when there was <+ turbidity at 48 h. In general, the cell extract of such cultures was tested for neuraminidase.

Preparation of cell extracts of Bacteroides species. In standard tests for neuraminidase production by Bacteroides species the 48-h or 96-h cultures were centrifuged (800 g for 60 min at 4°C). When it was desired to test the culture supernates, 5-ml samples of the centrifuged supernate were removed and stored at -20°C. The rest of the supernate was then removed from the packed cell deposit and the cells from the 10 ml culture were resuspended in 2 ml sterile normal saline. The resuspended cells were subjected to ultrasonic disintegration for 15-20 min at 0-1°C and these uncentrifuged cell extracts were stored at -20°C until assayed for neuraminidase.

A more concentrated bulk cell extract (P15) was prepared for tests of the effects of pH and Ca^{2+} on activity of B. fagilis NCTC9344 neuraminidase. An inoculum (2 ml) of a 48-h CMB culture was added to 200 ml PPW5 broth and incubated anaerobically at 37°C for 48 h. After centrifugation (2000 g for 1 h at 4°C) the packed cell deposit was resuspended in 5 ml sterile normal saline and disrupted by ultrasonic vibration for 30 min at 0-1°C; the uncentrifuged cell extract was stored at -20°C.

Neuraminidase assays and related procedures

Substrate preparations. Cohn fractions IV_1, IV_{1+4} and V were obtained from approximately 1000 donations of plasma from out-dated human blood by Cohn method 6 (Cohn et al., 1946); the fractionation procedures were done by Dr J.K. Smith, Scottish National Blood Transfusion Service, Protein Fractionation Centre, Ellen's Glen Road, Edinburgh EH17 7QT.

The following method was used to prepare NANA-rich glycoprotein (fraction VII) from the supernate of Cohn fraction V. A 120-litre volume of supernate from Cohn fraction V was titrated to pH 6.2 and cold (-25°C) ethanol was added until the final concentration of ethanol was 70% (v/v); the temperature of the solution was lowered to -10°C during the addition, and maintained for 24 h. The resulting white precipitate was removed by continuous-flow centrifugation in a refrigerated Sharples Super-centrifuge at 20,000 g and a flow rate of 40 litres/h. Approximately 700 g of moist precipitate was recovered and dissolved rapidly in 5 litres water. The solution was adjusted to pH 8.0 with 2M NaOH and pasteurised in a stainless steel vessel at 60°C for 10 h. The solution was cooled, titrated to pH 5.0 with dilute acetic acid and held frozen at -20°C. After some weeks it was thawed at 5°C, centrifuged at 4000 g for 1 h to remove insoluble protein, and filtered rapidly through Green's $904\frac{1}{2}$ fluted paper to remove floating lipoproteins. The solution was dispensed in vials, frozen and freeze-dried to a final vapour pressure of 13.33 N/m^2 (0.01 torr) at 20°C.

For information on the NANA content of different batches of FVII substrate see table I/II.

Total protein determinations. Protein concentration in the FVII substrate preparation was estimated by a biuret method (Gornall, Bardawill & David, 1949); a copper-free control reagent was used to correct extinction readings for the opalescence of the glycoprotein preparations.

Electrolytes. The concentrations of Na^+ and K^+ in substrate FVII were determined by flame photometry, Cl^- by a titrimetric method (Kit 830, Sigma London Chemical Co. Ltd) and Ca^{2+} by a fluorimetric method (Fingerhut, Poock & Miller, 1969).

Electrophoresis. Cellulose-acetate electrophoresis of substrate preparations was carried out on Sepraphore III medium (Gelman Instrument Co., Ann Arbor, Michigan, USA) at pH 8.6. Strips were stained for total protein (Ponceau S) and for glycoprotein (Kohn, 1968).

Gel filtration. Gel filtration of substrate preparations was at 5°C on Sephadex G-200 in 0.01M tris-borate/0.1M NaCl buffer, pH 8.6, in a glass column (2.5 x 80 cm) eluted at 20 ml/h. The extinction of the eluate was measured continuously at 254 nm in a flow-through analyser, and 5-ml fractions were collected for extinction readings at 280 nm. Certain fractions were also pooled and concentrated by ultrafiltration prior to electrophoresis on cellulose-acetate strips, and immunoelectrophoresis in agar.

Assay for N-acetyl neuraminic acid (NANA). Aminoff's thiobarbituric acid assay for free sialic acid as described by Cassidy et al. (1966) was followed. The reagents were: Reagent 1, 0.025M periodic acid (Koch Light Laboratories Ltd) in 0.25M H_2SO_4 (BDH Chemicals Ltd, Analar) at pH 1.2; Reagent 2, $NaAsO_2$ (BDH Chemicals Ltd) 2% (w/v) in 0.5M HCl (BDH Chemicals Ltd, Analar); Reagent 3, 0.1M 2-thiobarbituric acid (Koch Light Laboratories Ltd) in 0.1M NaOH (BDH Chemicals Ltd, Analar) adjusted to pH 9.0; and Reagent

4, butan-1-ol containing 12M HCl 5% (v/v). Pure synthetic NANA (Koch Light Laboratories Ltd) was used as a standard.

In routine tests, 0.5-ml samples were assayed in glass tubes (125 x 15 mm). Spectrophotometric readings of the organic phase at a wavelength of 549 nm were made with 4-ml glass, or disposable plastic, cuvettes (1-cm light path) in a spectrophotometer (Pye-Unicam Ltd, York St, Cambridge CB1 2PX; SP 600). The reference cell contained a "reagent blank" prepared by taking 0.5 ml distilled water through the assay procedure. When it was desired to analyse the absorbance curve for the assay product, the organic phase was examined in a scanning spectrophotometer (Pye-Unicam Ltd; SP 8000A), using the reagent blank in the reference cell.

Time-course studies of the release of NANA from substrate FVII by acid hydrolysis. Equal volumes (0.7 ml) of a known concentration of H_2SO_4 and of substrate dissolved in distilled water were mixed in screw-capped "bijou" bottles and promptly put into a water bath at 80°C. Reference bottles contained equal volumes of H_2SO_4 and of a solution of pure NANA in distilled water to demonstrate the extent of breakdown of NANA under the conditions of the test. The starting times were arranged so that the test periods of incubation ended simultaneously. The bottles were then rapidly cooled in two stages to 0-1°C, duplicate 0.5-ml samples were transferred to tubes and the assay for NANA was immediately performed. Time-zero (T_0) control tests were performed with chilled reagents, the mixture being held for only a few min at 0-1°C before the assay for NANA. Test results were corrected by subtraction of the appropriate T_0 control readings.

Tests for effects of culture materials on the NANA assay. Mixtures, prepared as for the standard neuraminidase assay but with NANA in place of substrate FVII, contained: 0.1 ml test substance; 0.15 ml sodium acetate buffer, pH 5.1; and 0.25 ml pure NANA (10 μg) in distilled water. Time-zero (T_0) tests were prepared with pre-cooled reagents. Each test value (E_{549}) was corrected by subtraction of the value obtained for control mixtures with buffer in place of NANA. Reference tubes containing only NANA in buffer were prepared by replacing the test substance with buffer, and each test result was compared with the appropriate reference value to show whether the test substance had interfered with the NANA assay.

When it was desired to demonstrate the extent of NANA breakdown (NAN-lyase activity) that might occur during incubation under the conditions of the neuraminidase assay, tests and controls were also incubated at 37°C for the appropriate period before assaying for NANA. Experiments were timed so that test periods of incubation ended simultaneously and all tests, including T_0 tests, were assayed for NANA in a single batch. Any breakdown of NANA during incubation of a test mixture can be detected by comparing the corrected values for the incubated test and the T_0 test. In standard assays for NAN-lyase activity in culture products of clostridial or Bacteroides strains, duplicate tests were incubated with NANA for 24 h at 37°C; culture products that did not reduce the assay value for 10 μg NANA (E_{549} c. 0.5) by 0.1 or more during 24 h incubation were regarded as having insignificant NAN-lyase activity.

Assay for neuraminidase activity. For the standard test, 0.5 ml of reaction mixture was prepared by adding the following volumes to glass tubes (125 x 15 mm): 0.1 ml of test enzyme preparation; 0.15 ml of 0.1M sodium acetate buffer, pH 5.1 (Cruickshank et al., 1975, p.88); and 0.25 ml of substrate FVII. See table I/III for concentrations of available NANA in standard assays with different batches of FVII substrate. Unless stated otherwise, dilutions of substrate or enzyme were made in acetate buffer, pH 5.1. Throughout the text the stated dilution of an enzyme preparation is that prepared for addition to the reaction mixture, but the concentration of substrate is normally given as the final concentration in the reaction mixture. Enzyme and substrate control tubes contained the equivalent volume of acetate buffer, pH 5.1, in place of the substrate and enzyme respectively. Reagents were normally held at room temperature before making the test mixtures, but were pre-warmed to 37°C when the period of incubation was to be less than 10 min. After incubation for the appropriate period at 37°C in a water bath, Reagent 1 was added to the mixture and the standard NANA assay was performed.

In studies of the time-course of the reaction with different concentrations of substrate and enzyme, standard tests were set up and incubated for graded periods of time. The timing of experiments was such that test periods of incubation ended simultaneously and all tests, including time-zero (T_0) control tests, were assayed for NANA in a single batch. For the T_0 control tests, the standard assay volumes of enzyme, substrate and buffer, pre-cooled to 0-1°C, were mixed in a tube held in an ice bath at 0-1°C;

the assay for NANA was begun immediately by adding Reagent 1 and transferring the mixture to a water bath at 37°C.

Each test and control assay was normally performed in duplicate and an average value for the extinction at 549 nm was calculated. The test reading was corrected to give a value equivalent to the amount of NANA released from the substrate during the incubation period by subtraction of either the sum of separate enzyme and substrate control readings or the value for the T_O control test; unless otherwise stated in the text, the results in these studies have been corrected by subtraction of separate enzyme and substrate control values. Reference assays of 10 µg of pure NANA in 0.5 ml distilled water were performed with each batch of assays. The results of neuraminidase assays are expressed in terms of spectrophotometric readings and are not routinely converted into equivalent concentrations of NANA because the presence of constituents of the culture medium may influence the reading obtained in the assay for NANA (see Section IIa).

Reference neuraminidase preparations. Our preparations of neuraminidase were standardised by comparison with a commercially available preparation of chromatographically purified C. perfringens (C. welchi) neuraminidase (type VI, N3001; Sigma London Chemical Co. Ltd); this batch had a declared activity of 1.1 unit/mg solid with NAN-lactose as substrate and 0.58 unit/mg with bovine submaxillary mucin as substrate, 1 unit liberating 1.0 µmole of NANA/min at pH 5.0 and 37°C from the test substrate (see Section IIf).

Vibrio cholerae Receptor Destroying Enzyme (RDE; Wellcome Reagents Ltd) was used as a source of V. cholerae neuraminidase.

Grading of clostridial neuraminidase activity. The relative amounts of neuraminidase produced by various clostridial strains and species in the supernates of the test cultures were graded according to the results of standard neuraminidase assays incubated for different periods of time at 37°C. All tests were performed in duplicate. The average value for the test assay (E_{549}) was corrected by subtraction of the sum of the values for separate substrate and enzyme control tests. Initial assays were incubated for 1 h before assay for free NANA. When the 1-h assay clearly showed neuraminidase activity, the assay was repeated with a 15-min incubation period. When the 1-h assay showed little or no activity, tests were incubated for 24 h in order to demonstrate the presence or absence of detectable neuraminidase activity. Tests that gave low values (E_{549} <0.5) in 24-h assays were further examined with the SP 8000A spectrophotometer to confirm that the absorption peak was at 549 nm, and at least one test from each neuraminidase-positive species was also examined in this manner, whether the assay values were high or low. In order to demonstrate clearly whether a low assay value was or was not due to the presence of NANA, it was occasionally necessary to read the absorption curve of the test mixture against a time-zero (T_0) control test, rather than the enzyme control, as reference sample.

The corrected results of neuraminidase assays were graded as follows: ++++, very high spectrophotometric values in 15-min assays (E_{549} >1.0); +++, NANA release clearly detectable in 15-min

assays (>0.1); ++, low values in 15-min assays (<0.1) but NANA release clearly detectable in 1-h assays (>0.1); +, not demonstrable in 1-h assays but high values (>0.5) in 24-h assays; ±, low values (<0.5 but >0.05) in 24-h assays and absorption peak clearly shown at 549 nm; -, no peak at 549 nm demonstrable in 24-h assays.

Grading of Bacteroides neuraminidase activity. Tests for neuraminidase activity in the cell extracts of Bacteroides strains were performed in the same manner as for the tests for clostridial neuraminidase and the results were graded on a similar scale. Since none of the Bacteroides samples contained as much neuraminidase as the clostridial samples graded ++++, 15-min assays were not performed. The corrected results of neuraminidase assays were graded as follows: +++, high values in the 1-h assays (E_{549} >0.5); ++, moderate NANA release clearly demonstrable in 1-h assays (>0.1); +, not demonstrable in 1-h assays but high values (>0.5) in 24-h assays; ±, low values (<0.5 but >0.05) in 24-h assays and absorption peak clearly shown at 549 nm; -, no peak at 549 nm demonstrable in 24-h assays.

Tests that gave low values (E_{549} <0.5) in 24-h assays were routinely examined to confirm that the absorption peak was at 549 nm; this was also done for at least one culture from each species even if the assay value was high. Culture products that gave very low or negative results (E_{549} <0.5) in 24-h neuraminidase assays were further examined to determine whether destruction of NANA by NAN-lyase activity might be interfering with the 24-h neuraminidase assay.

Buffers for pH studies. Sodium acetate buffers were prepared as described by Cruickshank et al. (1975, p.88); the pH range was extended to give buffers of higher pH by the use of progressively smaller volumes of acetic acid and correspondingly higher volumes of sodium acetate solution.

Tris-maleate buffer solutions were prepared as described by Diem & Lentner (1970, p. 280); the pH range was extended to give buffers of lower pH by the use of progressively smaller quantities of NaOH.

Studies on the effect of pH on C. perfringens neuraminidase. In order to ensure adequate buffering, tests were performed in a greater volume than for standard neuraminidase assays. The total reaction-mixture volume of 1.0 ml consisted of: 0.1 ml of enzyme preparation P9D2 diluted 1 in 2 in distilled water; 0.65 ml of the appropriate buffer; and 0.25 ml of substrate FVII(5) diluted in distilled water to give a final concentration in the reaction mixture of 143 µg/ml. Separate enzyme and substrate controls were prepared at each pH value by replacing the substrate and enzyme respectively with the equivalent volume of the appropriate buffer. Tests and controls were incubated for 30 min at 37°C and then assayed for NANA by the standard method.

The starting pH values of this series of tests were measured during a separate experiment. With pre-cooled reagents, test mixtures were prepared in tubes held at 0-1°C in an ice bath and the pH of each mixture was measured immediately. Small samples were brought to 37°C in a Radiometer BMS2 system and the pH values at 37°C were promptly read on a linked Radiometer PHM71 meter. The

pH values plotted for fig. II/7 are these measured values for the initial pH of the reaction mixture.

Studies on the effect of pH on the neuraminidase of B. fragilis. Assays were performed in a range of sodium acetate buffers in tests similar to those used with C. perfringens neuraminidase. The total reaction-mixture volume of 1.0 ml consisted of: 0.1 ml of the bulk cell extract (P15) of B. fragilis NCTC9344 diluted 1 in 40 in distilled water; 0.65 ml of the appropriate sodium acetate buffer; and 0.25 ml of substrate FVII(8) diluted in distilled water to give a final concentration in the reaction mixture of 160 µg NANA/ml. Separate enzyme and substrate controls were prepared at each pH value. Tests and controls were incubated for 30 min at 37°C before the assay for NANA.

The pH values shown in fig. V/1 are the initial values for the buffers added to the tests. Separate measurements were made of the initial pH values in sample reaction mixtures prepared with equivalent proportions but in 3-ml total volumes.

Studies on the effect of pH on C. perfringens NAN-lyase. The test mixtures contained: 0.1 ml of cell extract (P10) of C. perfringens strain L2Ab; 0.15 ml of the appropriate sodium acetate buffer; and 0.25 ml of pure NANA (10 µg) dissolved in distilled water. All assays were performed in duplicate and average values were used for calculation of results. The tests were incubated for 1 h at 37°C and the result (E_{549}) of the test at each pH value was corrected by subtraction of the assay value for the corresponding T_0 control test prepared with pre-cooled reagents.

Effect of Ca^{2+} and EDTA on neuraminidase activity. $CaCl_2.2H_2O$ (BDH Chemicals Ltd, Analar) or EDTA (ethylenediaminetetra-acetic acid disodium salt; BDH Chemicals Ltd, Analar) were added to the sodium acetate buffer, pH 5.1, used in the neuraminidase assay. The final concentrations of added Ca^{2+} or EDTA in the reaction mixtures were 3mM Ca^{2+}, 1mM Ca^{2+}, 1mM EDTA or 5mM EDTA. The test enzyme preparation (0.1 ml) and the acetate buffer containing Ca^{2+} or EDTA (0.15 ml) were mixed and held at 37°C for 30 min before addition of the substrate FVII (0.25 ml) and the start of incubation of the neuraminidase assay. Separate enzyme and substrate control tubes contained the equivalent volumes of acetate buffer in place of the substrate or enzyme respectively. Enzyme and substrate control tests also contained the appropriate amount of added Ca^{2+} or EDTA. All tests were performed in duplicate and average values were used in calculation of results. Each test result (E_{549}) was corrected by subtraction of the sum of the values for separate substrate and enzyme control tests.

Studies with antisera

Assay for serum anti-neuraminidase activity. Equal volumes of the test antiserum, diluted appropriately in saline, and of the C. perfringens neuraminidase preparation were mixed and held at room temperature for 30 min to allow neutralisation of the enzyme before the start of the standard assay for neuraminidase activity. Throughout the text the stated dilution of an antiserum is the initial dilution prepared for addition to the enzyme preparation; standard assays were performed with sera diluted 1 in 100. The

reaction mixtures contained: 0.05 ml neuraminidase preparation P9D3 or P9D4; 0.05 ml antiserum, diluted appropriately in saline; 0.15 ml acetate buffer, pH 5.1; and 0.25 ml substrate preparation FVII(7) or FVII(8). Enzyme and substrate control tubes contained the equivalent volume of acetate buffer, pH 5.1, in place of the substrate and enzyme respectively. Test and control assays were incubated for 30 min at 37°C before the start of the assay for NANA. Average values for duplicate test and control assays were used for calculation of the corrected assay value by subtraction of the sum of the values for the enzyme and substrate control assays. In a few experiments time-zero (T_0) tests were also performed; the assay for NANA was started immediately after addition of the substrate to the reaction mixture, with no period of incubation at 37°C. In general, however, T_0 assays are not recommended for correction of test results (see Section VIa).

Reference assays to determine the activity of the standard enzyme in the absence of antiserum were performed with enzyme that had been mixed with saline in place of antiserum during the neutralisation period. The degree of inhibition of the enzyme by the test antiserum was expressed as the percentage reduction in the test assay compared to the value given by the reference assay of the enzyme with no added antiserum.

Preparation of standard C. perfringens phospholipase-C (PLC). A 10-ml CMB culture of C. perfringens strain L2Ab was incubated aerobically at 37°C for 6 h. This starter culture was shaken to resuspend the organisms and the cooked-meat particles were allowed to settle before the broth was decanted and added to 1050 ml of

pre-reduced TGM broth. This bulk culture was incubated anaerobically at 37°C for 20 h; its purity was checked by microscopy and subculture to aerobic and anaerobic BA plates. After centrifugation (1200 g for 1 h at 4°C) the supernate was concentrated at 4°C in 7-cm Visking Cellophane tubing exposed to polyethyleneglycol MW 6000 for 3 days, followed by dialysis against running tap water for 2 days (final volume 30 ml). After centrifugation (10,000 g for 90 min at 4°C) the supernate was lyophilised in 4.5-ml volumes and stored at 4°C; this standard phospholipase-C preparation is referred to as PLC.

Tube tests for titration of phospholipase-C activity. The standard C. perfringens phospholipase-C preparation (PLC) was reconstituted and serial doubling dilutions were prepared with Michaelis' barbital sodium-acetate buffer, pH 6.8 (Diem & Lentner, 1962, p. 314). Tests were performed in glass tubes (75 x 11 cm) with 10% (v/v) lecithovitellin substrate as described by Holding & Collee (1971). Equal volumes (0.5 ml) of the test dilution of enzyme preparation and of lecithovitellin solution were mixed and incubated in a waterbath at 37°C for 24 h. Results were recorded as turbidity (+++), opalescence (++) or faint opalescence (+) by visual comparison with a control tube in which the test material was replaced by Michaelis' buffer, pH 6.8. The end-point was read as the tube that contained the minimum dose (MOD) that gave ++ opalescence; the titre of the reconstituted PLC preparation was found to be 64.

Assay for serum anti-phospholipase-C activity. The activity of the standard phospholipase-C preparation (PLC) was assayed in

tube tests in order to determine the minimum dose (MOD) that gave ++ opalescence in the reaction mixture after incubation at 37°C for 24 h (see above). Equal volumes (0.25 ml) of the test antiserum, serially diluted in Michaelis' buffer, pH 6.8, and of the PLC preparation diluted in the same buffer to contain 2 MOD, were mixed in the assay tubes and held for 30 min at room temperature to allow neutralisation. The standard assay for phospholipase-C activity was started by adding 0.5 ml of the lecithovitellin substrate and the reaction mixture was incubated at 37°C for 24 h. Reference samples contained saline in place of antiserum. The inhibitory activity of the test antiserum was expressed as the reciprocal of the highest dilution of serum that completely inhibited the activity of the standard amount of enzyme; dilutions of antiserum are expressed as the initial dilution prepared for addition to the test.

Equine antisera. C. perfringens type A antitoxin (CPA) for neuraminidase inhibition studies was obtained from Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS (C. welchi type A antitoxin, Wellcome Clostridial Diagnostic Antiserum: two batches were used; CPA1 = lot KO697, CPA2 = lot 9159B). The other C. perfringens sera (1863, 1881, 2748 and 5438) were provided by Prof. Collee; these experimental sera, raised in horses against C. perfringens type A culture products, were originally obtained from Wellcome Research Laboratories in 1963 and had been stored at 4°C. C. tetani antitoxin (ATS) was also an experimental equine antiserum originally obtained by Prof. Collee from Wellcome Research Laboratories.

Ammonium sulphate fractionation of PPW5 culture supernate of C. perfringens strain L2Ab (P12). More concentrated preparations of neuraminidase for rabbit immunisation studies were obtained by $(NH_4)_2SO_4$ fractionation of a further bulk PPW5 culture supernate (P12) of C. perfringens strain L2Ab. The P12 preparation was made by the same procedure as was used for the stock culture supernate P9 (see above) and stored at -20°C. Two batches of P12 culture supernate were subjected to $(NH_4)_2SO_4$ fractionation as described by Cassidy et al. (1966) in order to obtain the precipitates produced at 50% and 85% saturation. All procedures were carried out in the cold (0-4°C). After centrifugation (20,000 g for 60 min) the precipitates were redissolved in distilled water before concentration in a stirred ultrafiltration cell (Model 52; Amicon Ltd, Upper Mill, Stonehouse, Gloucestershire GL10 2BJ) at 45 lb/in^2 with a PM10 membrane (10,000 MW cut-off). The samples were assayed for protein (Lowry et al., 1951) and for neuraminidase and were stored at -20°C until used for immunisation of rabbits.

Two batches of P12 culture supernate were fractionated in this way. For batch A, 500 ml of the bulk P12 preparation was thawed and fractionated directly. Before addition of $(NH_4)_2SO_4$ to batch B, the P12 preparation (580 ml) was centrifuged (27,500 g for 60 min at 4°C) to remove a slight precipitate and then dialysed against tap water at 4°C to remove peptone medium constituents that interfered with the assay for protein in the starting material. For the relative amounts of neuraminidase and protein in the various fractions, see Section VIb.

C. perfringens type A neuraminidase (Sigma) for rabbit immunisation. For immunisation studies, further batches were obtained of the chromatographically purified C. perfringens neuraminidase used previously as a reference neuraminidase preparation (see above: Sigma London Chemical Co. Ltd; type VI, N3001). Three batches were used, with the following declared specific activities: batch 1, 2.1 units neuraminidase activity/mg protein; batch 2, 5.4 units/mg; batch 3, 6.5 units/mg (1 unit liberates 1.0 μmole NANA/min from NAN-lactose substrate at pH 5.0 and 37°C).

Procedure for immunisation of rabbits. Adult New Zealand White rabbits (2-3 kg) of either sex were immunised with C. perfringens neuraminidase (see Section VIc for details of antigen preparations and schedules of immunisation). Samples of blood (usually 5 ml) were taken before the first injection (pre-inoculation sample) and at intervals during the course of immunisation; the serum was separated and held at -20°C. The following adjuvant preparations were used: Freund's complete adjuvant (Difco Laboratories, 0638-60-7); Freund's incomplete adjuvant (Difco Laboratories, 0639-60-6); and aluminium hydroxide (Alhydrogel; Miles Laboratories Ltd, PO Box 37, Stoke Poges, Slough, Berkshire SL2 4LY). For each injection the antigen preparation was emulsified with an equal volume of adjuvant in a Sorvall Omni-mixer (Du Pont UK Ltd, Biomedical Products Division, Wedgwood Way, Stevenage, Hertfordshire SG1 4QN). The total volume given in a single injection was 2-4 ml; subcutaneous injections were divided and given in four separate sites. Batches of sera, diluted 1 in 100 in saline, were assayed in standard anti-

neuraminidase assays in order to monitor the response of each rabbit. A final collection of blood was made by cardiac puncture when the animal was killed.

Gel-diffusion analysis of rabbit serum. Serum was examined for the presence of antibody by gel diffusion in a Petri dish (Cruickshank et al., 1975, p.241). The plates contained 1% (w/v) ionagar (Ionagar No. 2; Oxoid Ltd) and were examined after being held at room temperature for 48 h.

Guinea pig pathogenicity studies

Guinea pigs. Normal guinea pigs of either sex and varied markings were bred in the Animal House in this Department. Animals weighed 350-500 g and were allowed free access to food and water during the experiment.

Calcium chloride. A 15% (w/v) solution of $CaCl_2.6H_2O$ (BDH Chemicals Ltd, Analar) in distilled water was autoclaved at 121°C for 15 min and used as sclerosing agent for promoting infection in guinea pigs.

Adrenaline. Adrenaline tartrate 1 in 1000 (Evans Medical Ltd, 891 Greenford Rd, Greenford, Middlesex UB6 0HE) was diluted 1 in 10 in sterile saline; this solution (100 µg adrenaline/ml) was held in the dark until used in animal inoculation studies.

Preparation of bacterial inocula. The following procedure was used to prepare washed cell suspensions containing known

numbers of log-phase organisms of various clostridial species. Each strain was freshly grown in CMB medium from freeze-dried stock and its purity was tested by 24-h aerobic and anaerobic subculture on BA plates. A second CMB culture (10 ml) from an inoculum of 0.1 ml of the initial culture was incubated anaerobically for 18 h at 37°C. A further bottle of CMB medium (20 ml) was held uninoculated with the cap loose in the same anaerobic jar, so that it was pre-warmed and pre-reduced when the jar was opened next morning. In order to produce an actively growing log-phase culture, 0.2 ml of the 18-h culture was added with minimal delay to the 20 ml of pre-reduced medium; the jar was promptly made anaerobic and put in a waterbath at 37°C. After incubation for 2 h, the culture was shaken to resuspend the organisms and the cooked-meat particles were allowed to settle before removal of the broth. The packed cells (18,000 g for 10 min at 4°C) were washed once in 20 ml sterile saline and the cells were resuspended in 1 ml sterile saline. In some experiments this washed cell suspension was used undiluted to provide a high challenge dose for inoculation into the animal; in others, the number of organisms was rapidly estimated by a total count and the suspension was diluted appropriately in sterile saline to give the desired number of cells in the inoculum.

The purity and identity of the organism in the suspension prepared for each inoculum was confirmed by subculture to BA plates incubated aerobically and anaerobically at 37°C for 24 h. The number of bacteria present in the inoculum was measured with minimal delay by performing total and viable cell counts with appropriate dilutions of the washed cell suspension. (For

bacterial counting methods, see above.) Throughout the text, the challenge dose cited is the number of organisms in the inoculum as determined by total count (cfu). In general, when the results of the viable counts became available next day they were very close to the original total counts. Difficulties were experienced in some early experiments when dilutions were prepared in saline and the viable counts were delayed while the animals were inoculated; these counts suggested a 100-fold or greater loss of viability in the inoculum. However, this was shown to be an artefact when pre-reduced NB medium was substituted for saline as diluent and the viable counts were promptly performed during the time when the animals were also being inoculated.

Inoculation of bacteria into guinea pigs. The washed bacterial suspension was injected into the left hind leg of the test animal. In experiments with $CaCl_2$ as sclerosing agent, 0.2 ml of 15% $CaCl_2$ was injected into the muscle mass of the left thigh 3 h before the freshly prepared bacterial inoculum (0.2 ml of washed cell suspension) was injected into the same site. Uninfected control animals received an injection of 0.2 ml sterile saline 3 h after the injection of $CaCl_2$.

When adrenaline was used for initiating infection, the adrenaline and the challenge dose of bacteria were mixed in the same syringe just before inoculation; the 0.2-ml inoculum contained 0.1 ml of bacterial suspension mixed with 0.1 ml of adrenaline solution (10 µg adrenaline). Control animals received an inoculum containing 0.1 ml of adrenaline but with 0.1 ml sterile saline in place of the bacterial suspension.

Classification of outcome of bacterial challenge. The animals were observed daily for up to 7 days and the course of infection was noted. Some animals developed overwhelming infection and died during the first or second day; the actual time of death was often not more accurately known. Animals that were judged to be severely ill or moribund (immobile, cold, with ruffled fur) were killed at that stage by a blow on the head. Surviving animals usually had only local signs in the inoculated leg (swelling, stiffness, flexion) and were killed after 6-7 days. At post-mortem examination the degree of infection in the thigh muscles was noted. In general it was possible to classify the result of challenge for each animal in one of the following broad categories: G = acute gangrenous infection with extensive thigh muscle necrosis and haemorrhagic oedema spreading to the anterior abdominal wall, resulting in severe illness or death within 1-2 days; N = severe local damage with extensive necrosis of thigh muscle but animal survived for 3 days or more; LA = animal survived with signs limited to the inoculated leg and a large abscess was found at post-mortem examination; MA = similar but less severe infection with a well-defined medium-sized abscess found in the inoculated thigh muscles; SA = limited swelling and stiffness in the inoculated leg and a small localised abscess found; NAD = minor, sometimes transient, signs in the inoculated leg, but no abnormality detected post mortem. These abbreviations are used in the tables in Section VII; where significant differences from these categories occurred they are described in the text.

Sampling of guinea pig tissues and reisolation of challenge strain. Tissue samples were taken from both legs at the post-mortem examination; samples of liver were also taken from some animals. Where the thigh muscles were intact, as large a sample as possible was taken. With animals that had developed gangrene, the necrotic tissue and oedematous exudate were taken along with remaining portions of muscle; a sterile syringe was used for collection of liquid samples. In some animals there was a localised abscess within the thigh muscle; these samples contained varying amounts of abscess content, abscess wall and surrounding intact muscle.

The samples were taken with sterile instruments and were placed in sterile screw-capped glass bottles, but it was difficult to avoid some degree of contamination from the overlying skin. A few animals developed superficial lesions over the affected thigh; samples from these were particularly liable to contamination or superinfection. Samples were put at -20^{o}C as soon as possible (and always within 1 h) and were kept in the cold thereafter in order to prevent further multiplication of infecting or contaminant organisms. Gram films were made with impression smears or swabs from the tissue samples of the inoculated limb to show the presence of morphologically typical organisms. In order to reisolate and confirm the identity of the infecting strain, cultures were made from the inoculated thigh muscle samples on BA plates incubated aerobically and anaerobically for 24 h; in some experiments, CMB medium and EYA plates were also inoculated. Animals were excluded from the study if the reisolated strain differed from that inoculated, if more than one strain of Clostridium was recovered,

or if another neuraminidase-producing contaminant was isolated; these exclusions were few.

Homogenisation of tissue samples for neuraminidase assays. The samples were promptly weighed; sodium acetate buffer, pH 5.1, was added in the ratio of 3 ml buffer to 1 g tissue and the bottles were put at -20°C. Thawed samples, held in an ice-water bath at 0-1°C, were later treated with a homogeniser (MSE Scientific Instruments) for 2-10 min as required. The homogenised samples were centrifuged (800 g for 30 min at 4°C) and the supernates were held at -20°C. Samples (0.1 ml) were assayed for neuraminidase in standard assay mixtures incubated for 1 h; repeat assays were incubated for 24 h if clearly positive results were not obtained. The results of assays for neuraminidase in the tissue extracts were graded by the same scale (+++ to -) as was used for grading assays with Bacteroides culture products (see above).

Tests for C. sordelli β-toxin production. Freeze-dried stock of each test strain was inoculated into 10 ml CMB medium and incubated anaerobically at 37°C for 24 h; purity was checked by aerobic and anaerobic subculture on BA plates. Aliquots (2 ml) of the CMB cultures were centrifuged (5500 g for 20 min at room temperature) and 0.1 ml of each culture supernate was given by intradermal injection to depilated albino guinea pigs. A sample of supernate from uninoculated CMB was included as a control. Animals were observed for 48 h. After 24 h β-toxin action was seen as a large (1-2 cm diameter) oedematous zone with a central purple-brown necrotic ulcer; this increased considerably in size by 48 h.

There was no visible reaction to β-toxin-negative samples after 24 or 48 h.

Tests for protective effect of antisera in guinea pigs. Each animal was given 1 ml of a 1 in 2 dilution of the test antiserum in sterile saline by intraperitoneal (IP) injection 20 h before challenge with a standard number of washed cells of C. perfringens strain L2Ab. Control animals were given 1 ml of saline in place of the antiserum. The procedure for challenge of the guinea pigs was as described above, with an inoculum containing a challenge dose known to be the minimum lethal dose for the experimental conditions employed, i.e. with either adrenaline or $CaCl_2$ as initiating agent. The outcome of infection was recorded as previously described.

RESULTS

I. EVALUATION OF A GLYCOPROTEIN FRACTION (FVII) PREPARED FROM POOLED HUMAN PLASMA AS SUBSTRATE FOR THE ASSAY OF CLOSTRIDIUM PERFRINGENS NEURAMINIDASE

Ia. Preparation and characterisation of glycoprotein fractions

The α_1-acid glycoprotein (orosomucoid) of human plasma is normally one of the waste products of large-scale plasma fractionation. Various glycoprotein-containing fractions were prepared from out-dated human plasma (see Materials and Methods) and assessed for suitability for use as substrate in assays for neuraminidase activity. In preliminary experiments, the glycoproteins of Cohn fractions IV_1 and IV_{1+4} were studied. When these fractions were redissolved in water they were found to contain material rich in N-acetyl neuraminic acid (NANA) that withstood pasteurisation at pH 8. However, the preparations were very turbid and contained a material that acted as a chromogen in the spectrophotometric assay for NANA before hydrolysis; the absorption spectrum of the product had a peak at c. 530 nm that overlapped to an unacceptable degree with that derived from pure NANA (peak absorbance at 549 nm). Unsuccessful attempts were made to remove the interfering material by absorptive filtration, isoelectric precipitation, ultracentrifugation and fractionation with polyethyleneglycol MW 4000.

The one-stage preparation, fraction VII (FVII), proved much simpler and the product had none of the disadvantages of the fraction IV derivatives. The procedure used for preparation of

batch FVII(5) is detailed in Materials and Methods. Several other such large-scale preparations were made and used in the course of the present studies; the technique used for later batches was essentially the same, differing only in minor details of scale, resolution volume and the volume dispensed for freeze-drying. Each batch was readily soluble in water, even in concentrations exceeding 50 mg/ml. When a vial of batch FVII(5) containing 18.4 mg dry powder was dissolved in 1 ml water the solution contained; 12.1 mg protein, 46 μmole Na^+, <1 μmole K^+, 8 μmole Cl^-, and 0.42 μmole Ca^{2+}.

Cellulose-acetate electrophoresis of FVII (fig. I/1) separated it into a minor zone with the mobility of serum albumin and a major, rather broad, "inter-α" zone with a mobility intermediate between the α_1 and α_2 serum globulins. The albumin zone stained very poorly for glycoprotein, while the inter-α zone stained quite intensely. Because the relative dye-binding characteristics of these proteins were unknown, it was not possible to assess the proportion of the total protein migrating in each zone.

The preparation was separated into two peaks by gel filtration on Sephadex G-200 (fig. I/2). The first peak was excluded from the gel, and therefore consisted of proteins with MW >200,000. Eluates from each peak were reconcentrated and examined by cellulose-acetate electrophoresis and immunoelectrophoresis.

The high-MW peak contained approximately 65% of the applied protein. It consisted of glycoprotein occupying the more slowly migrating region of the broad inter-α zone seen in the whole

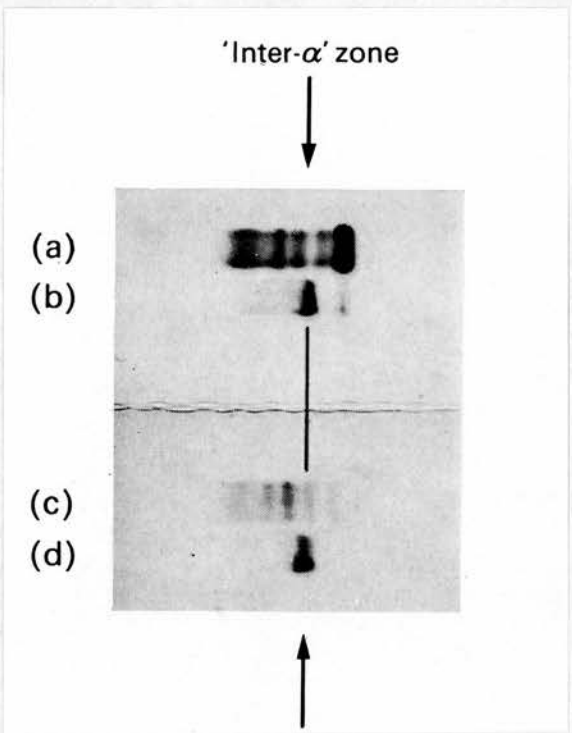


Fig. I/1 - Separation of glycoprotein preparation FVII by cellulose-acetate electrophoresis. Samples: (a) and (c), human serum; (b) and (d), fraction FVII(6). Strips (a) and (b) stained for total protein; strips (c) and (d) stained for glycoprotein. (From Fraser & Smith, 1975; included in Appendix.)

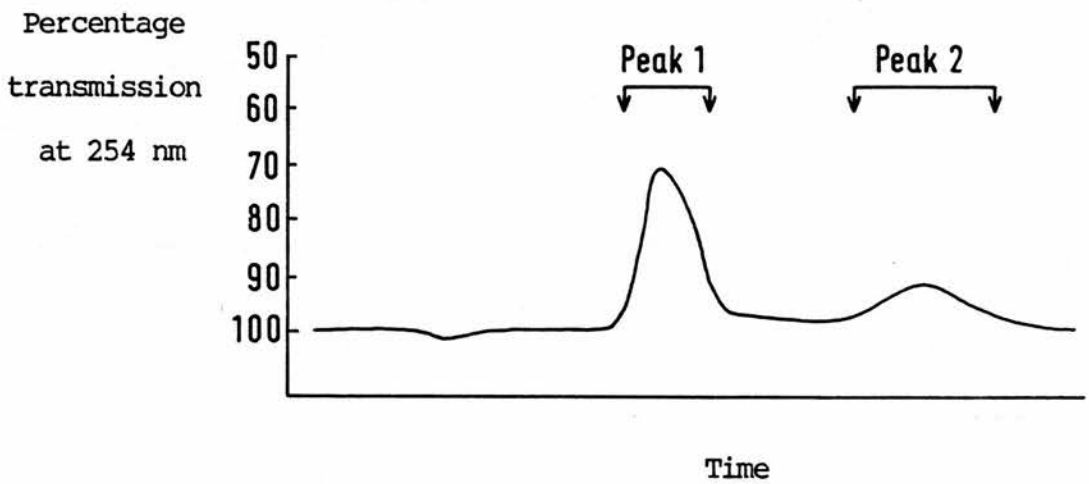


Fig. I/2 - Separation of glycoprotein FVII on Sephadex G-200. Sample: 2 ml containing 70 mg FVII(6). Eluate monitored at 254 nm. Horizontal scale represents time, which is linearly proportional to eluate volume. After separate concentration of each peak, peak 1 was found to contain mainly aggregated glycoprotein and peak 2 to contain mainly monomeric albumin and α_1-acid glycoprotein.

preparation; there was also a trace of polymerised albumin, detectable only by immunoelectrophoresis.

The low-MW peak contained approximately 35% of the applied protein. It consisted largely of monomeric albumin, but also contained a little glycoprotein occupying the faster-migrating region of the broad inter-α zone. Both gel filtration peaks, i.e. both the faster- and slower-migrating regions of the inter-α zone, were rich in orosomucoid detectable by immunoelectrophoresis.

Ib. Release of NANA from substrate FVII

Release of NANA from FVII by acid hydrolysis. When samples of NANA are heated at 80°C in 0.02M H_2SO_4 there is usually very little breakdown of the NANA within 2 h. Fig. I/3 shows the effect of subjecting samples of FVII to the same treatment. Separate test mixtures were prepared and incubated at 80°C for graded periods of time; the timing was such that test periods of incubation ended simultaneously and all tests were assayed for NANA in a single batch (see Materials and Methods). Test results (E_{549}) were corrected by subtraction of the values for the appropriate time-zero (T_0) control tests. There was progressive release of NANA reaching a steady maximum between 1 and 2 h and there was excellent correlation between the values obtained with the two test concentrations of FVII. When a similar experiment was performed with incubation in the same concentration of acid but at 100°C (Yamashina, 1956) there was considerable breakdown of NANA (fig. I/4). Further tests showed that the use of stronger acid (0.04M H_2SO_4) did not release more NANA from FVII but did also produce some breakdown of NANA after 2 h at 80°C.

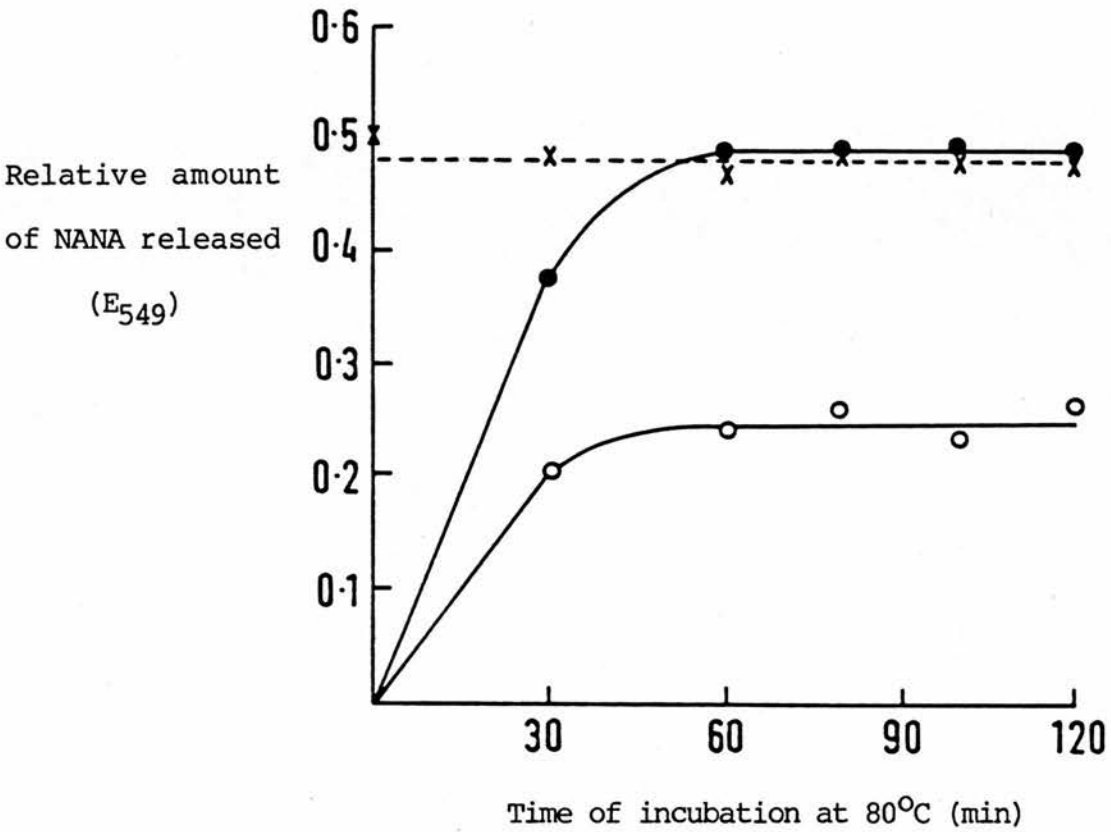


Fig. I/3 - Release of N-acetyl neuraminic acid (NANA) from FVII substrate at two test concentrations by mild acid hydrolysis (final concentration 0.02M H_2SO_4) at 80°C. The relative amount of NANA released is plotted as the corrected extinction value of the assay product at 549 nm. Concentrations in acid-hydrolysis mixtures: ●—●, FVII(5) 0.46 mg/ml; ○—○, FVII(5) 0.23 mg/ml; x--x, NANA 20 µg/ml.

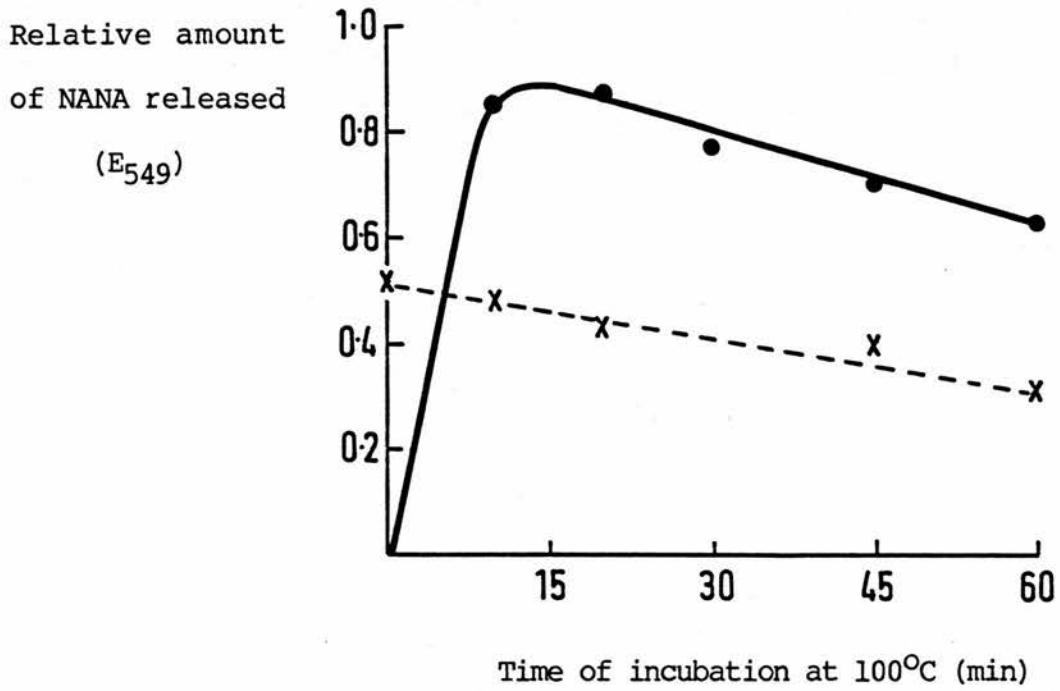


Fig. I/4 - Release of NANA from FVII substrate by mild acid hydrolysis (0.02M H_2SO_4) at 100°C. The relative amount of NANA released is plotted as the corrected extinction value of the assay product at 549 nm. Concentrations in acid-hydrolysis mixtures: ●—●, FVII(5) 0.92 mg/ml; x--x, NANA 20 µg/ml.

The total amount of NANA that can be released from substrate batches FVII(5) and FVII(6) was estimated by incubating appropriate concentrations in 0.02M H_2SO_4 at 80°C for 2 h (table I/I). The extinction value (E_{549}) obtained on assay of NANA incubated in acid under the same conditions was used for converting the test values into equivalent concentrations of NANA. Substrate batch FVII(5) contained 4.7% dry weight of NANA, and batch FVII(6) a little less.

Release of NANA from FVII by C. perfringens neuraminidase. The total amount of NANA that can be released from substrate FVII by C. perfringens (C. welchi) neuraminidase was determined by incubation of appropriate concentrations of FVII(5) with undiluted stock enzyme preparation P9D2 (see Materials and Methods). Standard tests were prepared and incubated at 37°C in acetate buffer, pH 5.1, for graded periods of time; the timing was such that test periods of incubation ended simultaneously and all tests were assayed for NANA in a single batch. Appropriate T_0 control tests were used for correction of test results. When reaction mixtures were incubated for periods up to 2 h there was progressive release of NANA to a steady maximum after 80 min (fig. I/5); there was excellent correlation between the amounts of NANA released from the two test concentrations of substrate. Comparison of the results given in figs I/3 and I/5 shows that a similar amount of NANA was released from FVII by C. perfringens neuraminidase as was released by the acid hydrolysis procedure.

Ic. Enzyme-substrate kinetic studies

Initial velocity of neuraminidase reaction with varying concentrations of substrate FVII. Enzyme preparation P9D2 was used at

TABLE I/I

Estimation of NANA content of two batches of human glycoprotein substrate FVII by acid hydrolysis

Batch of substrate FVII	Concentration of FVII in 0.02M H_2SO_4 (µg/ml)	Spectrophotometric reading after incubation at 80°C for 2 h[a] (E_{549})	Concentration of NANA in test mixture[b] (µg/ml)	NANA as percentage of dry weight of FVII
FVII(5)	460	0.495	21.4	4.7
FVII(6)	890	0.865	37.4	4.2

[a] The spectrophotometric value (E_{549}) is an average result obtained from duplicate 2-h assay readings corrected by subtraction of the value for the corresponding T_0 test (see Materials and Methods).

[b] A sample of pure NANA at a final concentration of 20 µg/ml in the acid gave a reading (E_{549}) of 0.462 after incubation at 80°C for 2 h.

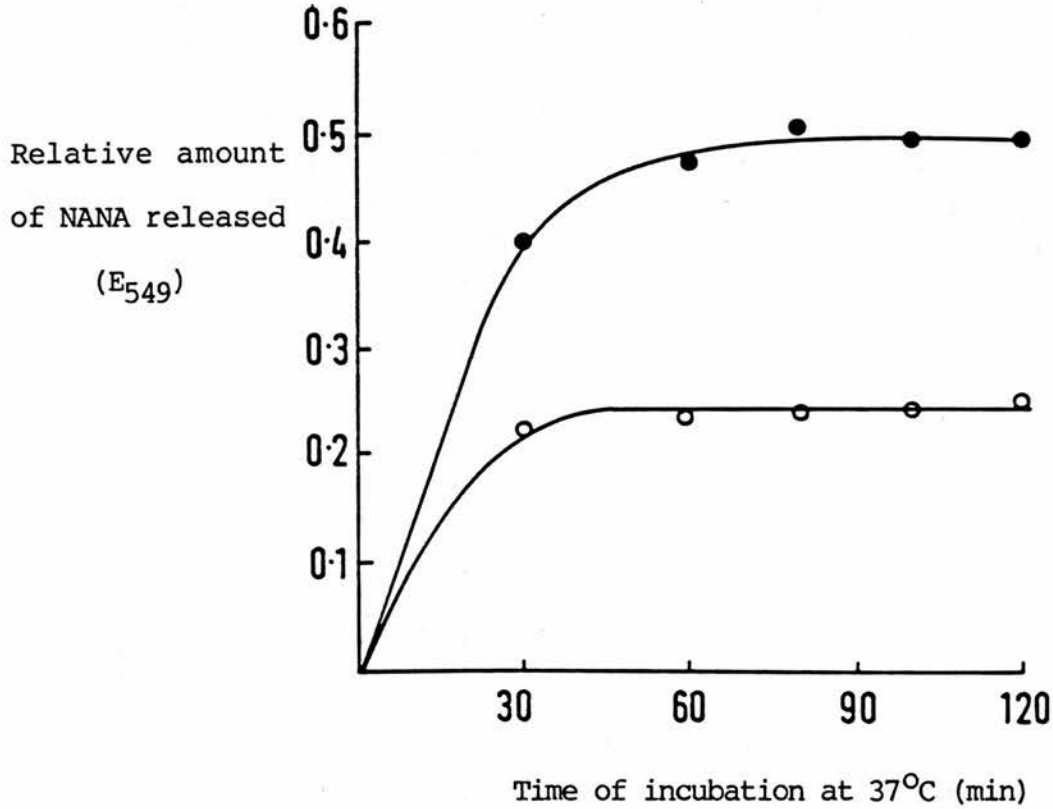


Fig. I/5 - Release of NANA from FVII substrate at two test concentrations by Clostridium perfringens neuraminidase. Substrate incubated with enzyme preparation P9D2 at 37°C, pH 5.1. Concentrations of FVII(5) in reaction mixtures: ●—●, 0.46 mg/ml; o—o, 0.23 mg/ml.

a dilution of 1 in 2 in the acetate buffer, pH 5.1, and a range of concentrations of substrate FVII(5) was prepared in the same buffer. Reaction mixtures were prepared for the standard neuraminidase assay and were incubated at 37°C for graded periods of time before the NANA assay; T_0 control tests were used for correction of test readings. Fig. I/6 shows the plots of values for the amount of NANA released by the enzyme. These are linear for the first 10 min for all test concentrations of substrate, and a value for the initial reaction velocity (v) at each substrate concentration (s) was derived from this portion of the graph. Fig. I/7 shows the relationship of (v) to (s). Maximum velocity (Vmax) occurred at substrate concentrations of c. 3 mg/ml; the standard concentration of batch FVII(5) used in subsequent experiments was 3.07 mg/ml. The data given in fig. I/7 allow calculation of the values plotted in fig. I/8. The slope of the line = $^1/_{Vmax}$; from this the value for Vmax was calculated to be 0.26 µg NANA released from FVII/min. The $^s/_v$ intercept (0.6) indicates the value for $^{Km}/_{Vmax}$; thus Km = 0.16 mg/ml for this substrate (formulae from Dixon & Webb, 1964, chap. 4).

Initial velocity of neuraminidase reaction with varying concentrations of enzyme preparation. The final concentration of substrate FVII(5) was 3.07 mg/ml in the reaction mixture, and enzyme preparation P9D2 was diluted in acetate buffer, pH 5.1, to give test concentrations of 10, 25, 50, 75 and 100% (v/v). Standard neuraminidase assays were carried out with the different reaction mixtures incubated at 37°C for 10, 20, 30 or 40 min before the NANA assay. Appropriate T_0 control tests were used for

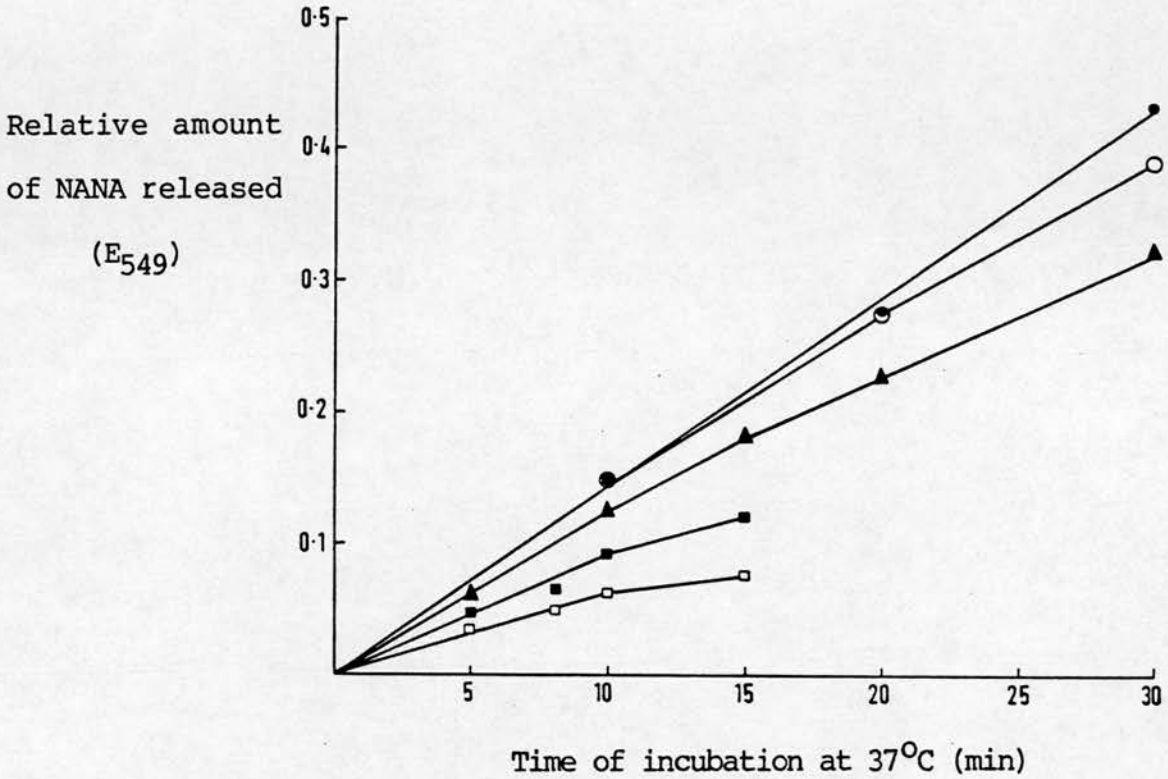


Fig. I/6 - Velocity of Clostridium perfringens neuraminidase reaction when a standard amount of enzyme preparation P9D2 was incubated with graded concentrations of substrate at pH 5.1. Concentrations of substrate FVII(5) (mg/ml of reaction mixture): ●—●, 9.2; ○—○, 3.07; ▲—▲, 0.92; ■—■, 0.23; □—□, 0.12.

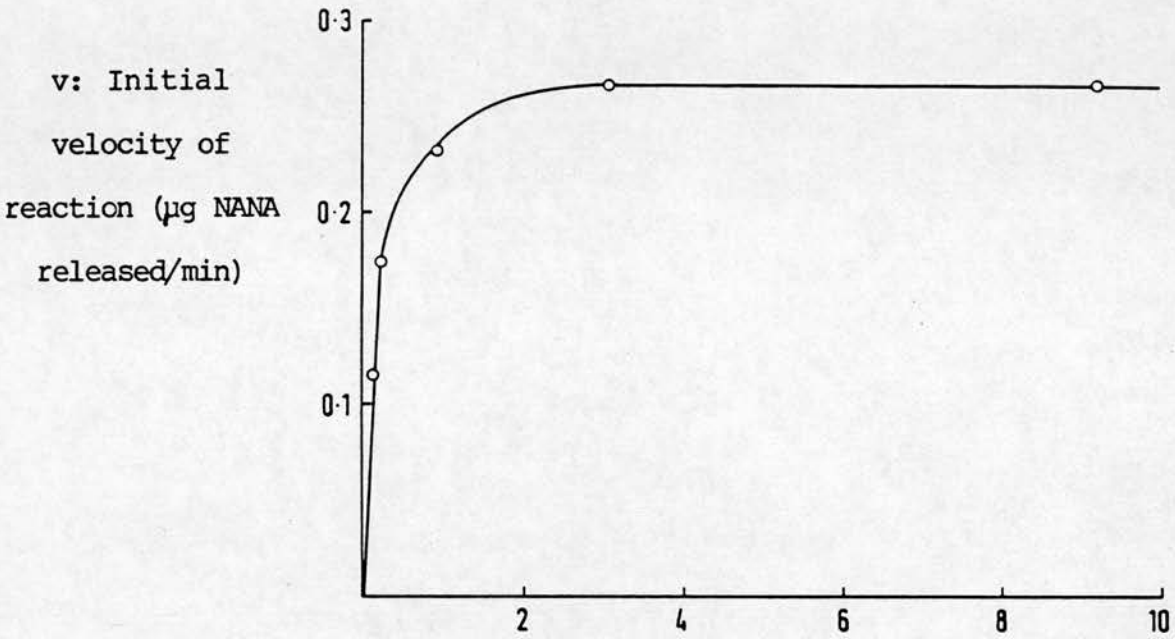


Fig. I/7 - Relationship of initial reaction velocity (v) of neuraminidase reaction to concentration of substrate (s). The value for v is calculated from the observed release of NANA during the first 10 min (fig. I/6); a reference assay of 10 µg pure NANA gave a spectrophotometric reading (E_{549}) of 0.536 and the data given in fig. I/6 have been converted accordingly. The observed V max is 0.26 µg NANA/min.

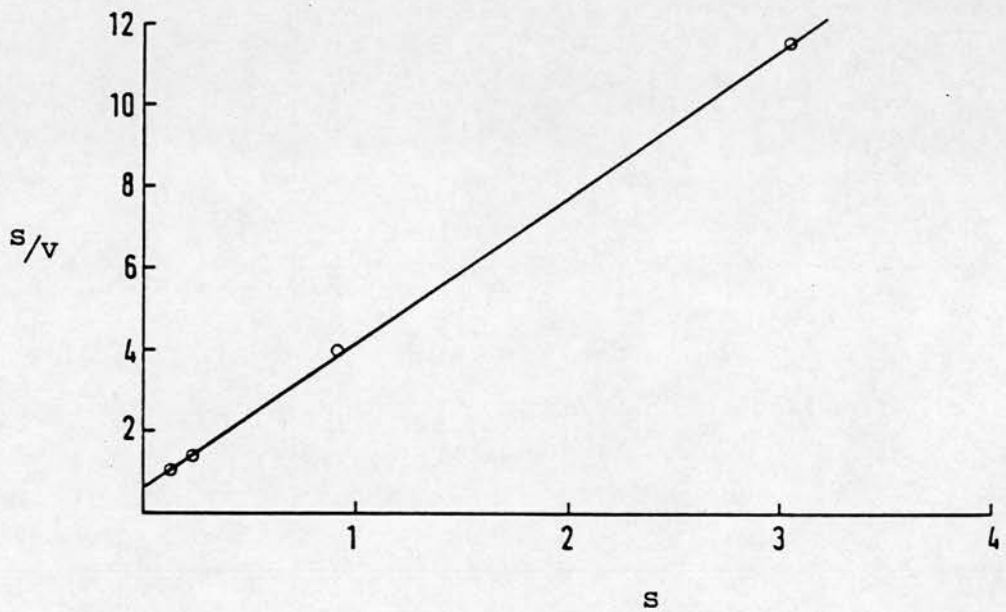


Fig. I/8 - The relationship of the substrate concentration (s) to the value obtained for the quotient $\frac{s}{v}$ (data from fig. I/7).

correction of test results. Fig. I/9 shows that the rate of release of NANA was proportional to the concentration of the neuraminidase preparation when the reaction mixtures were incubated for periods up to 40 min. Thus, using a 15-min assay, we could measure amounts of neuraminidase approximately 0.1-3 times that of the test P9D2 preparation; this is equivalent to a range of c. 2-50 milliunits/ml (see table II/V for activity of P9D2). More concentrated neuraminidase preparations should be diluted before the assay so that the results fall into the reliable range.

Experiments similar to that described above were used to follow the release of NANA from the same amount of substrate by lower concentrations of enzyme P9D2 during longer periods of incubation at 37°C. Fig. I/10 shows that the plot of the release of NANA from FVII remained linear for 4 h with test concentrations of P9D2 from 1 to 10%. Fig. I/11 extends the period of observation and shows that the plot of the release of NANA from the substrate is linear for 26 h with suitable dilutions of the enzyme; as little as 0.5% of the enzymatic activity of P9D2 (i.e. c. 0.1 milliunit/ml) can be reliably detected with a 24-h test.

Id. Comparative data on various batches of substrate FVII

Data on the amount of NANA that can be released by mild acid hydrolysis from substrate batches FVII(5) and FVII(6) were given in table I/I. Similar experiments were performed to estimate the NANA content of each subsequent batch of substrate prepared and information on the various batches of FVII used in the course of the present studies is summarised in table I/II. The NANA content

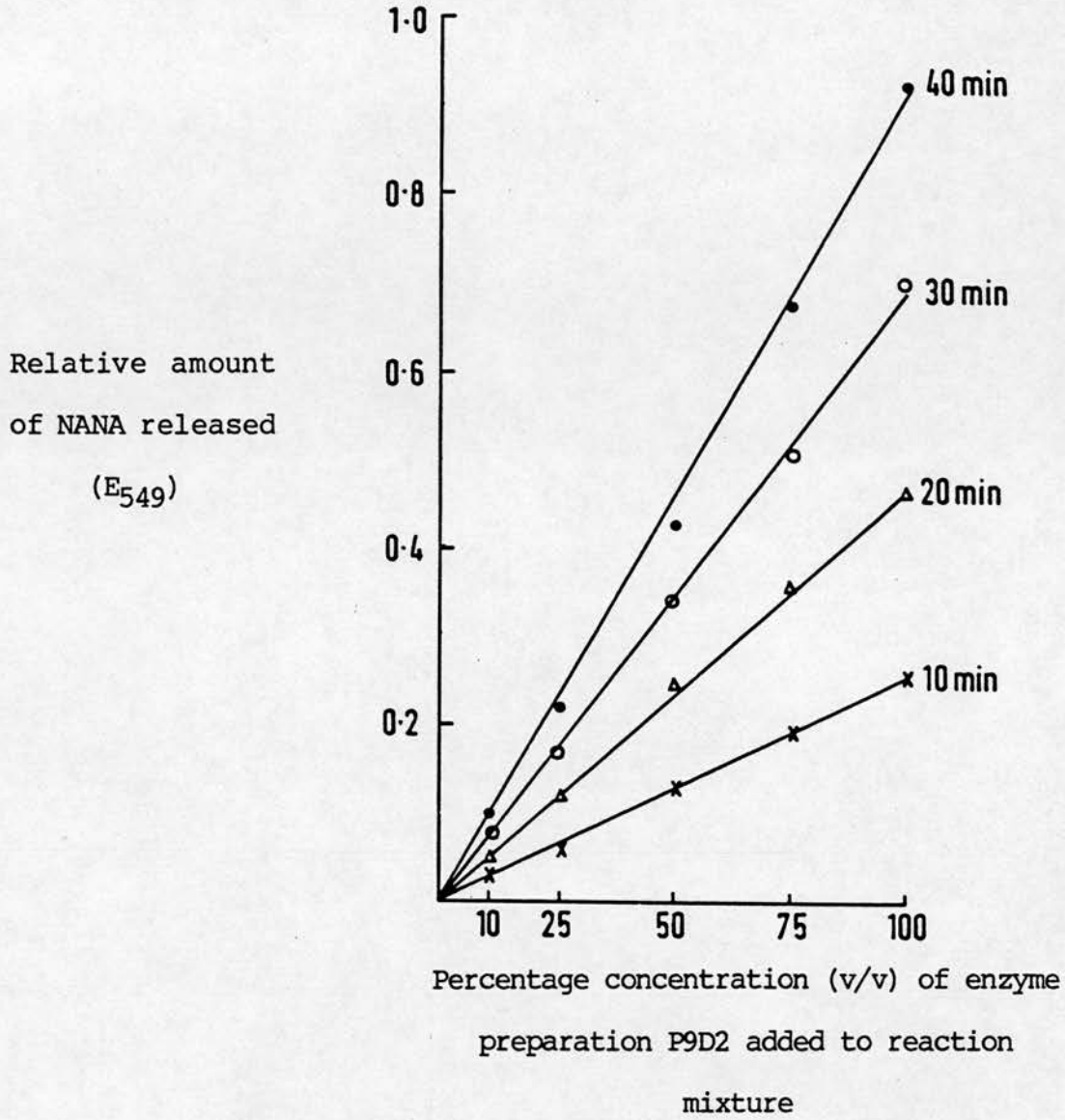


Fig. I/9 - Amounts of NANA released at different times from a standard amount of substrate FVII(5) by graded amounts of Clostridium perfringens neuraminidase. Period of incubation at 37°C, pH 5.1 (min): ●—●, 40; o—o, 30; Δ—Δ, 20; x—x, 10.

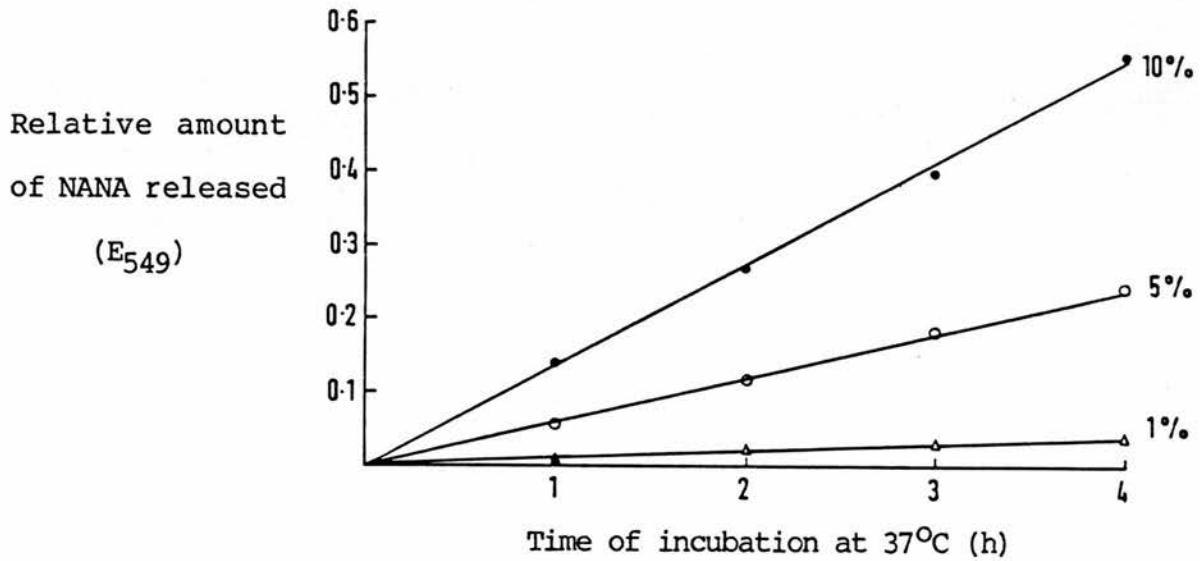


Fig. I/10 - Release of NANA from substrate FVII(5) by low concentrations of Clostridium perfringens neuraminidase during incubation at 37°C, pH 5.1, for 4 h. Percentage concentration (v/v) of enzyme preparation P9D2 added to reaction mixture: ●—●, 10; o—o, 5; Δ—Δ, 1.

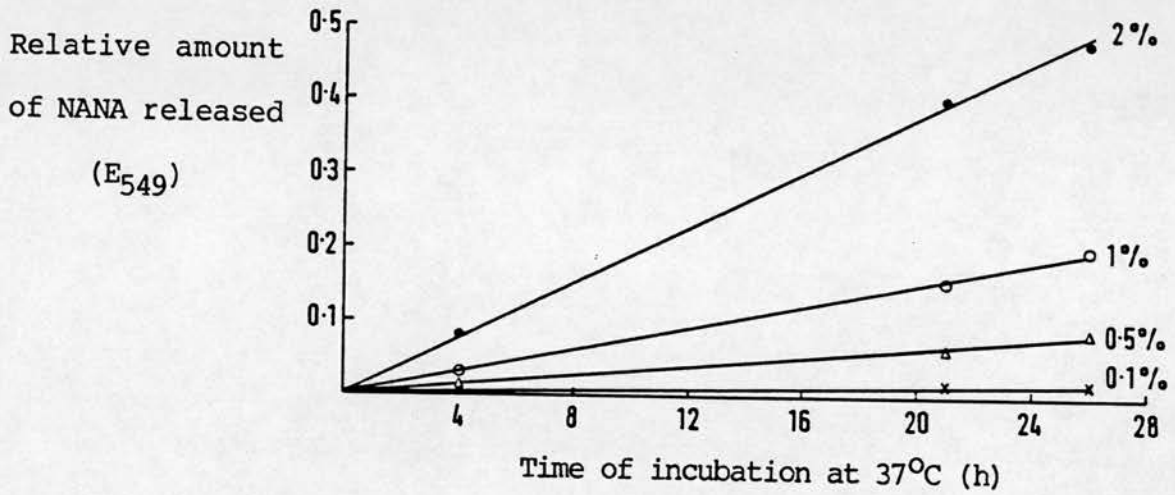


Fig. I/11 - Release of NANA from substrate FVII(5) by very low concentrations of Clostridium perfringens neuraminidase during prolonged incubation at 37°C, pH 5.1. Percentage concentration (v/v) of enzyme preparation P9D2 added to reaction mixture: ●—●, 2; o—o, 1; Δ—Δ, 0.5; x—x, 0.1.

TABLE I/II

Comparative data on the NANA content of five batches of substrate FVII estimated by acid hydrolysis

Batch of substrate FVII	Weight of FVII/vial (mg)	Weight of NANA/vial[a] (mg)	NANA as percentage of dry weight of FVII
FVII(5)	18.4	0.86	4.7
FVII(6)	178.0	7.49	4.2
FVII(7)	472.8	5.59	1.2
FVII(8)	135.1	5.12	3.8
FVII(11)	132.0	4.59	3.5

[a] See table I/I for method of NANA determination by acid hydrolysis. The value obtained for the test concentration of substrate was used to calculate the total amount of available NANA/vial for each batch of substrate.

of the different batches of substrate was in the range 3.5-4.7%, with the exception of batch FVII(7) which contained only 1.2% NANA.

Initially, the concentration of substrate added to neuraminidase assays was expressed in terms of the total weight of the FVII preparation (e.g. see fig. I/6). However, since different batches of substrate vary a little in their content of glycoprotein, the substrate concentration is better expressed in terms of the amount of available NANA present in the reaction mixture. The standard concentration of substrate FVII(5) for use in neuraminidase assays was determined from the experiment shown in figs I/6 and I/7 and was fixed at 3.07 mg/ml; this is equivalent to a concentration of 143 µg releasable NANA/ml. The concentrations of subsequent batches of FVII used in neuraminidase assays were standardised to give 140-170 µg NANA/ml in the final reaction mixture (table I/III).

The performance of each batch of substrate was compared in standard neuraminidase assays with undiluted enzyme P9D3. Assays performed with the substrate concentrations shown in table I/III were incubated for 10, 20 and 30 min; T_0 control tests were used for correction of the results. Separate substrate control tests (see Materials and Methods) assayed at T_0 and after incubation at 37°C for 30 min all gave low values (E_{549} <0.04) that did not rise during incubation at 37°C; further tests confirmed that there was no increase in value when incubation of samples of these batches of substrate was continued for 24 h.

Fig. I/12 shows the plot of the assay values obtained. Substrate batches 5, 6, 8 and 11 gave comparable results with assay values (E_{549}) in the range 0.25-0.30 after 10 min, 0.48-0.54 after

TABLE I/III

Concentration of available NANA present in standard neuraminidase assays with five batches of substrate FVII

Batch of substrate FVII	Concentration of available NANA in standard reaction mixtures[a] (µg/ml)
FVII(5)	143
FVII(6)	166
FVII(7)	140
FVII(8)	146
FVII(11)	153

[a] Concentration of available NANA in standard assay mixtures (see Materials and Methods) calculated from values for NANA content (see table I/II) with reference to dilution procedures used for each batch of substrate.

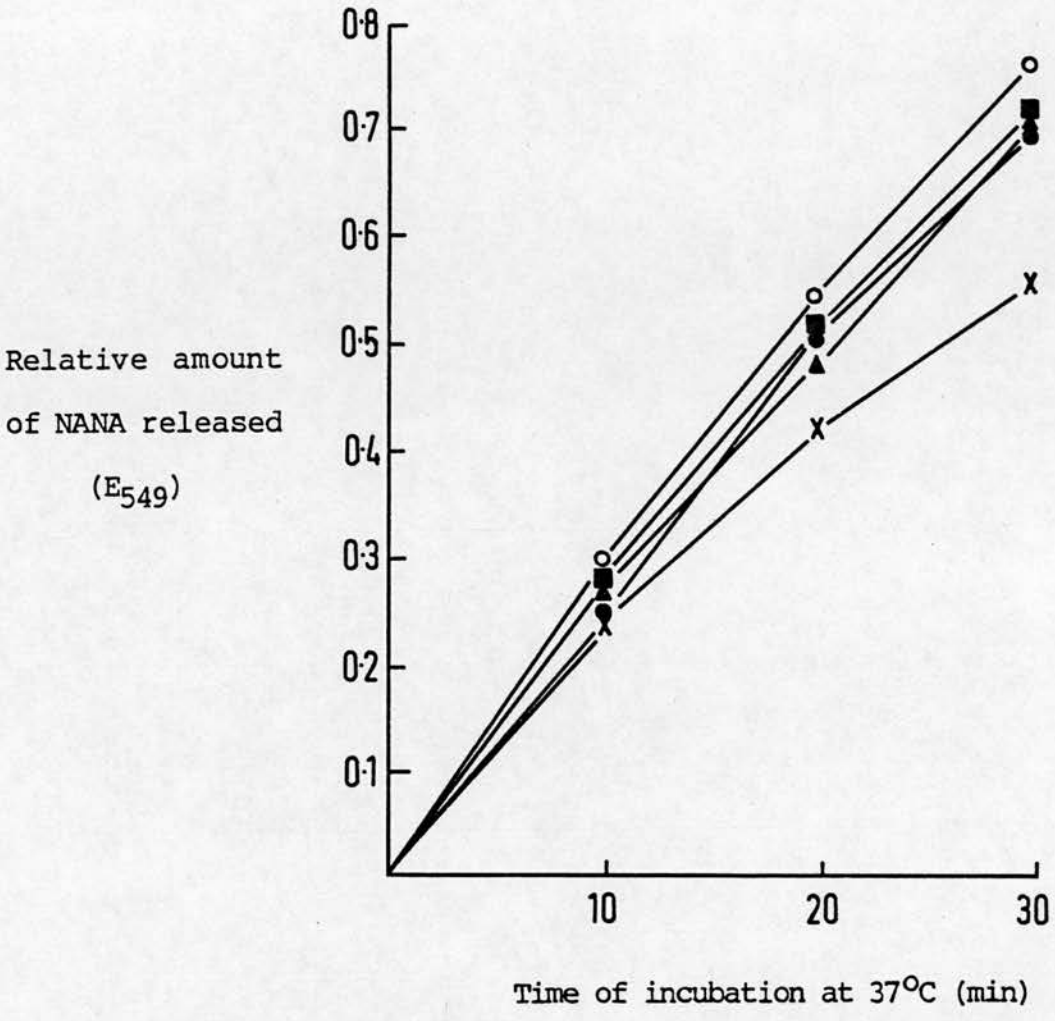


Fig. I/12 - Rate of release of NANA from different batches of substrate FVII by a standard amount of Clostridium perfringens neuraminidase preparation P9D3 during incubation at 37°C, pH 5.1. Batch of substrate tested: ●—●, FVII(5); ○—○, FVII(6); x—x, FVII(7); ▲—▲, FVII(8); ■—■, FVII(11).

20 min and 0.69-0.75 after 30 min incubation. The values for the 20-min assays were not quite double those for the 10-min assays but there is an approximately linear correlation up to values of c. 0.5; readings above this level become progressively less accurate and will tend to underestimate the enzyme activity. Substrate batch FVII(7) gave comparable results in the 10-min assay, but thereafter gave lower values than the other batches tested. Assay values with batches 5, 6, 8 or 11 should thus be comparable; assays with batch FVII(7) are liable to give rather lower values, especially at higher readings.

II. DEVELOPMENT OF STANDARD PROCEDURES FOR ASSAY OF NEURAMINIDASE ACTIVITY

IIa. Studies on the assay for NANA

Reference assays with pure NANA. Fig. II/1 shows the curve obtained when the assay product for 10 μg pure NANA dissolved in distilled water is examined with the scanning spectrophotometer; the peak absorbance (c. 0.52) is at 549 nm. Similar assays with 10 μg NANA dissolved in the standard assay buffer, pH 5.1, confirmed that the assay values were unaffected by the presence of the buffer.

The assay of graded amounts of pure NANA gives a linear plot for spectrophotometric values (E_{549}) up to c. 0.7 (e.g. see fig. II/3); readings above this level are likely to be a little high and give an overestimate of the amount of NANA present. When values higher than 0.7 are obtained in an assay, an approximate indication of the amount of NANA present can be obtained by diluting the assay product before reading the spectrophotometric value; however, the correlation between direct readings with the undiluted product and readings after dilution in Reagent 4 (acid butan-1-ol) follows a similar curve and may give rise to a similar inaccuracy. When accuracy is important, samples should be diluted before assay so that the results fall in the reliable range; for neuraminidase assays it may be more convenient to shorten the incubation period in order to achieve this.

Solutions of pure NANA in distilled water or in the standard assay buffer, pH 5.1, are stable for many months at -20°C. Batches of tubes containing 10 μg pure NANA in 0.5 ml distilled water were

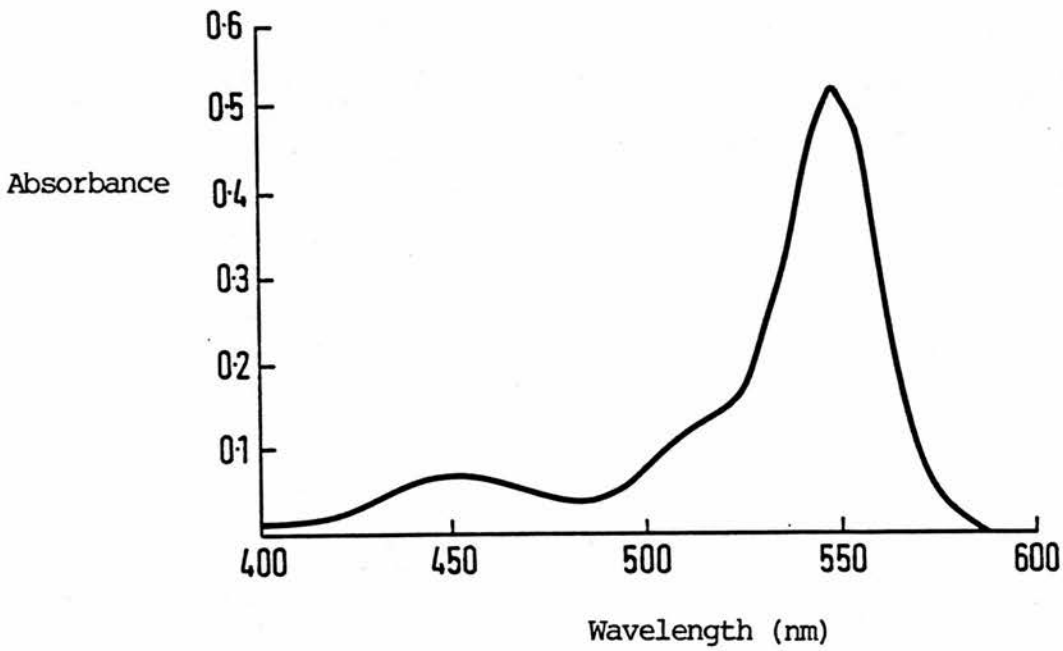


Fig. II/1 - Absorbance curve of the assay product for NANA. Sample put through the assay: 10 µg pure NANA in 0.5 ml distilled water. Absorbance curve read on the SP 8000A scanning spectrophotometer with reagent blank in reference cell (see Materials and Methods).

prepared at intervals and held at -20°C for use as reference standards with subsequent neuraminidase assays. The results of the NANA assays were reasonably consistent for series of assays performed over a number of days or weeks (e.g. see Section IIe); however, over a longer period of time there may be greater differences. Variation in assay values for the NANA standards may be attributable to a number of factors, including different batches of assay reagents, slight variations in volumes of reagents added and minor inaccuracies in incubation times at various stages during the assay. The commonest cause of low readings encountered appeared to relate to differences in performance of the $NaAsO_2$ in Reagent 2.

In general, assay of the 10 µg NANA standards gave a reading (E_{549}) between 0.45 and 0.50. On occasion, the results might be as high as 0.55; the assays were not accepted if the value fell below 0.40. Duplicate 10 µg NANA standards were included with each batch of neuraminidase assays; the average value for these tests was used when it was desired to express results of an experiment in terms of rate of NANA release rather than as comparative spectrophotometric values.

Effect of culture materials on the assay for NANA. Experiments were performed to study the effects of sterile proteose peptone water (PPW5) medium on the assay for NANA. The total test volume (0.5 ml) consisted of: 0.1 ml undiluted PPW5 broth; 0.25 ml containing 10 µg NANA dissolved in distilled water; and 0.15 ml acetate buffer, pH 5.1. Control samples contained PPW5 broth alone or NANA alone, with the NANA or PPW5 respectively replaced by

acetate buffer. A reagent blank, prepared by taking 0.5 ml distilled water through the NANA assay, was used in the reference cell for the absorbance curves shown in fig. II/2(a). The assay product for the sample containing PPW5 broth alone contained a chromogen with a peak absorption at c. 530 nm that gave rise to significant absorption at 549 nm (c. 0.12). The absorption curve for 10 µg NANA was distorted in the presence of PPW5 broth so that the peak fell at c. 540 nm and there was a high shoulder at c. 530 nm. Fig. II/2(b) shows the absorbance curve for the same product when read with the assay product for the PPW5 broth alone in the reference cell; this confirms that the true peak for NANA occurs at 549 nm. However, it is also apparent that the height of the NANA peak is rather lower (c. 10%) than for the reference sample of 10 µg NANA in the absence of PPW5 broth.

Fig. II/3 shows the results of a similar experiment when graded amounts of pure NANA were assayed in the presence and in the absence of PPW5 broth. The test values (E_{549}) obtained when NANA was assayed in the presence of PPW5 broth were corrected by subtraction of the value for the control assay for PPW5 alone (0.128); these corrected values were distinctly lower than the values for equivalent amounts of pure NANA over the whole range tested.

The extent of the depression of the assay value for the standard test dose of NANA (10 µg) by constituents of PPW5 broth may vary in different batches of the medium; in general, a reduction of 5-10% was found. Constituents of other media, e.g. Todd-Hewitt broth (THB), may produce a similar, though usually less marked, effect on the assay for NANA. The effect may also be seen

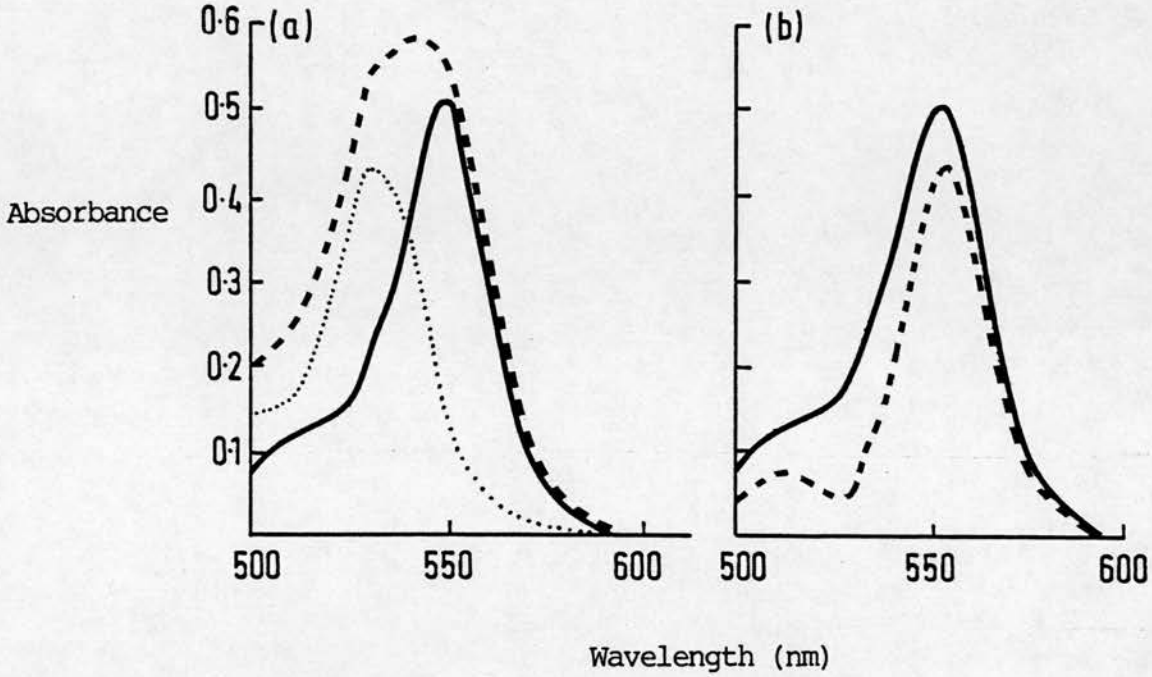


Fig. II/2 - Effect of PPW5 broth on the absorbance spectrum for NANA. See text for details of samples taken through the assay for NANA: - - -, PPW5 + 10 µg NANA; ····, PPW5 alone. Absorbance curves read in SP 8000A scanning spectrophotometer with (a) reagent blank and (b) assay product for PPW5 alone in reference cell. The solid line indicates the curve obtained with assay of 10 µg NANA read with reagent blank in the reference cell and is included in both (a) and (b).

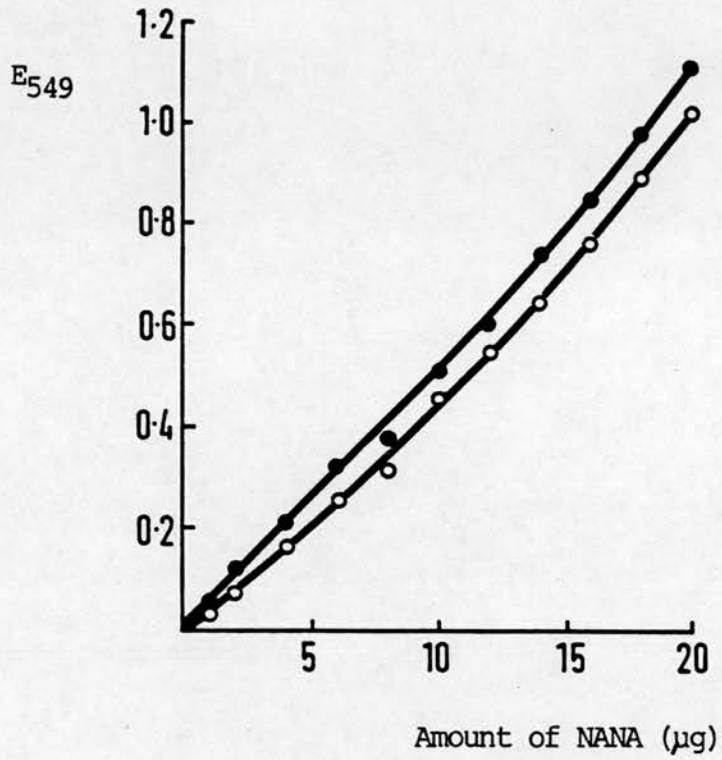


Fig. II/3 - Effect of PPW5 broth on the assay for NANA. Absorbance of assay product (E_{549}) for graded amounts of NANA in acetate buffer, pH 5.1: o—o, NANA + PPW5; ●—●, NANA alone (average of duplicate spectrophotometer readings).

with supernates of cultures in these media, e.g. the PPW5 culture supernate (P9) used as stock neuraminidase preparation in these studies; however, the interfering substance(s) can be removed by dialysis (e.g. see table II/V). The effect is seen predominantly in test materials that give high enzyme control values (E_{549}), and there is a rough correlation between the level of the enzyme control test and the extent of depression of the NANA assay value. The effect may be avoided by working with dialysed culture preparations; it is important to make allowance for it in neuraminidase assays with crude culture products, and also in assays for NAN-lyase activity (see Section IIb). With occasional cultures in PPW5 medium, the absorption curve for small amounts of NANA was distorted when the standard enzyme control test was used in the reference cell; this could be avoided by the use of a time-zero (T_0) test as the reference preparation (see Section IVc).

IIb. Tests for N-acetyl neuraminate pyruvate-lyase (NAN-lyase) activity

Since the neuraminidase assay depends on the detection of NANA released by the enzyme, results may be influenced by factors that interfere with the assay for NANA; in particular, NANA might be broken down by NAN-lyase present in the test material. An assay procedure was developed to determine whether the presence of such factors in culture products might be responsible for falsely low or negative results in tests for neuraminidase.

Cell extract and culture supernate prepared from a bulk PPW5 culture of C. perfringens strain L2Ab (P10; see Materials and Methods) were tested for the ability to break down NANA under

conditions equivalent to those of the standard neuraminidase assay. Test mixtures, containing 0.1 ml test material, 0.15 ml acetate buffer, pH 5.1, and 0.25 ml of a solution containing 10 µg pure NANA dissolved in distilled water, were incubated at 37°C for periods up to 4 h before the assay for NANA. Test results were corrected by subtraction of the value for control tests containing buffer in place of the NANA, and the results were expressed as the percentage change from the corrected assay value for an unincubated time-zero (T_0) test. The results plotted in fig. II/4 show that the cell extract produced a progressive reduction in the assay value for NANA, with c. 80% of the test dose of NANA destroyed after incubation for 4 h. By contrast, the culture supernate produced no NANA breakdown during this period of incubation, and this suggests that NAN-lyase remained cell-associated in this culture.

A further experiment was performed in order to assess the effect of pH on the activity of C. perfringens NAN-lyase. Similar test mixtures of P10 cell extract and NANA were prepared, but using a range of sodium acetate buffers from pH 3.6 to pH 6.6 (see Materials and Methods). The tests were incubated for 1 h at 37°C and then put through the assay for NANA. Test results were expressed as the percentage reduction from the assay value for a T_0 test with acetate buffer at pH 5.1 (E_{549} = 0.450); preliminary studies confirmed that the assay value for pure NANA was constant over this pH range. Fig. II/5 shows a plateau of enzyme activity above pH 6.0; as the pH range tested did not extend above pH 6.6 the peak activity was not determined, but these results are consistent with the broad optimal pH range of 6.8-8.0 reported by

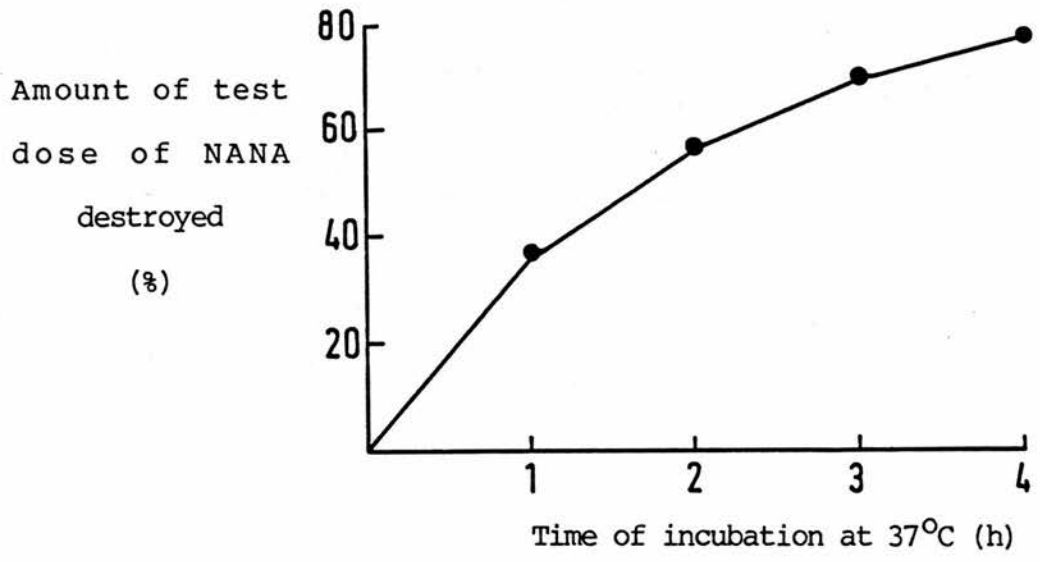


Fig. II/4 - Demonstration of NAN-lyase activity in Clostridium perfingens cell extract. Test preparation (P10 cell extract) incubated with 10 µg NANA in acetate buffer, pH 5.1, at 37°C for periods up to 4 h before the assay for NANA. Test results corrected by subtraction of enzyme control value (E_{549} = 0.010); assay value expressed as percentage reduction from value in T_o test (E_{549} = 0.420).

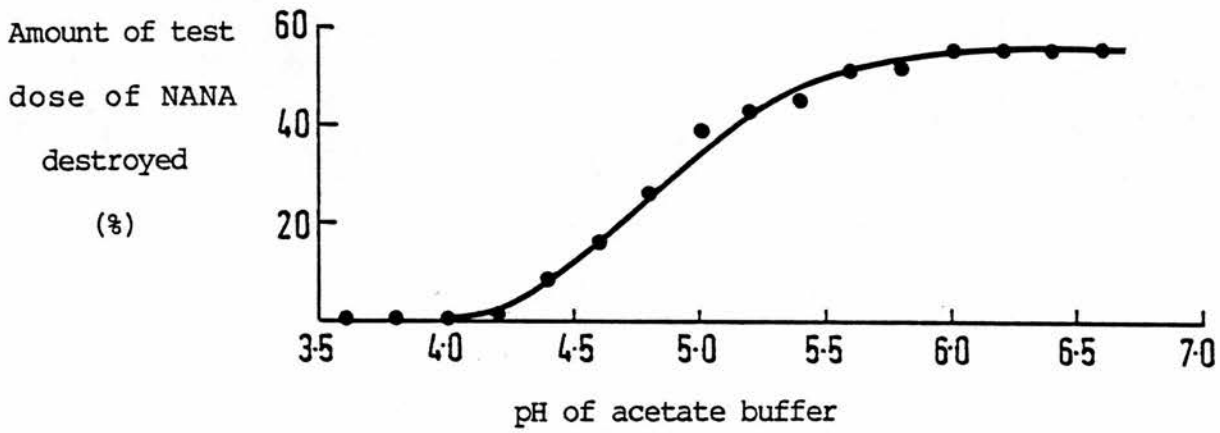


Fig. II/5 - Effect of pH on the activity of Clostridium perfringens NAN-lyase activity. Cell extract P10 incubated at 37°C with 10 µg NANA in sodium acetate buffers for 1 h before assay for NANA.

Brunetti et al. (1963). NAN-lyase activity was reduced by c. 20% in the acetate buffer, pH 5.1, used in the standard neuraminidase assay, and was completely inhibited below pH 4.2.

Similar tests were used to evaluate the extent by which various preparations used in these studies might interfere with the accurate determination of NANA under conditions equivalent to those used in the standard assay for neuraminidase. Table II/I gives the detailed results of tests with sterile PPW5 broth and with the stock culture filtrates of C. perfringens in this medium, P9 and P9D2. Tests were incubated at 37°C in acetate buffer, pH 5.1, for periods up to 1 h before the start of the assay for NANA. The corrected value for the T_0 assay of each test mixture shows the extent of the depression of the true value for NANA before the start of incubation at 37°C. Sterile PPW5 broth reduced the assay reading by c. 10% and the undialysed P9 preparation reduced it by c. 25%; the dialysed preparation P9D2 produced less than 5% depression of the assay value. There was, however, no further depression of the assay values when the mixtures were incubated at 37°C for 1 h. Similar tests confirmed that there was no significant breakdown of NANA even when incubation was prolonged to 24 h. It was also confirmed that the FVII substrate used in neuraminidase assays did not affect the assay value for NANA in 24-h tests.

The absence of NAN-lyase activity in the supernates of PPW5 broth cultures P9 and P10 confirms that the C. perfringens enzyme is normally cell-associated. However, some enzyme may occasionally be released into the culture supernate, e.g. the

TABLE II/I

Effect of culture materials on NANA determination in time-zero mixtures and in mixtures incubated at 37°C before assay

Test mixture[a]	Time of incubation at 37°C (min)	Spectrophotometric reading[b] (E_{549})		
		Test	Control	Corrected value[c]
NANA alone	0	0.506	...	0.506
	15	0.509	...	0.509
	60	0.502	...	0.502
NANA + PPW5	0	0.620	0.175	0.445
	15	0.605	0.171	0.434
	60	0.607	0.173	0.434
NANA + P9	0	0.596	0.232	0.364
	15	0.583	0.221	0.362
	60	0.602	0.225	0.377
NANA + P9D2	0	0.500	0.017	0.483
	15	0.481	0.018	0.463
	60	0.505	0.016	0.489

[a] Test mixtures contained 10 µg NANA in 0.25 ml distilled water, 0.1 ml test substance and 0.15 ml acetate buffer, pH 5.1; NANA alone = NANA + 0.25 ml buffer. PPW5 = sterile broth; P9 = Clostridium perfringens neuraminidase preparation; P9D2 = dialysed P9 (see Materials and Methods).

[b] Average of duplicate assay readings; ... = not applicable.

[c] Test readings corrected by subtraction of the control value (0.1 ml test substance + 0.4 ml buffer).

results presented in table III/XI show slow breakdown of NANA by the supernate of a similar culture of the same strain. It is important, therefore, to consider the possibility of NANA-destroying activity in all assays for neuraminidase that give low-positive or negative results.

IIc. Standard procedures for assay of C. perfringens neuraminidase

Control assays for correction of test results. The standard procedure for the neuraminidase assay is described in Materials and Methods. Separate enzyme and substrate control tubes, containing the acetate buffer in place of the substrate and enzyme respectively, were normally included for each assay and incubated for the same period of time as the test assay tube. The test spectrophotometric reading (E_{549}) was corrected by subtraction of the sum of the values for the separate enzyme and substrate control assays.

When it was desired to study the rate of release of NANA from substrate in progress experiments (e.g. see figs I/6 and I/9), the use of unincubated time-zero (T_0) control tests with pre-cooled reagents (see Materials and Methods) proved more convenient and more reliable than the use of separate enzyme and substrate control assays for correction of test results. The data given in table II/II show that the assay value for T_0 tests with mixtures of enzyme preparation P9 and substrate FVII (E_{549} = 0.246) was equivalent to the calculated value for the sum of the separate T_0 enzyme and substrate control assays (0.252). This also held good for the tests with P9D3 (T_0 assay = 0.040; enzyme control + substrate control = 0.036). There was no increase in value in

TABLE II/II

Comparison of values for time-zero neuraminidase assays with values for separate enzyme and substrate control tests incubated at 37°C for periods up to 24 h

Test mixture[a]	Spectrophotometric reading[b] after incubation at 37°C for stated period (E_{549})			
	T_0	1 h	18 h	24 h
P9 alone	0.226	0.226	0.220	0.218
P9 + FVII(5)	0.246	...	...	...
P9D3 alone	0.010	0.013	0.009	0.009
P9D3 + FVII(5)	0.040	...	...	...
FVII(5) alone	0.026	0.031	0.031	0.033

[a] Test mixtures contained enzyme (0.1 ml) and/or FVII(5) substrate (0.25 ml); the final reaction-mixture volume (0.5 ml) was reached by addition of appropriate amounts of acetate buffer, pH 5.1 (see Materials and Methods). P9 = *Clostridium perfringens* enzyme preparation; P9D3 = dialysed P9.

[b] The spectrophotometric reading is the average result for duplicate assays incubated at 37°C for the appropriate period; T_0 tests were not incubated at 37°C before the assay for NANA (see Materials and Methods): ... = not tested.

enzyme or substrate control tests after incubation at 37°C in the assay buffer for periods up to 24 h. Test results with these reagents may therefore be corrected by subtraction of either the sum of the values for separate enzyme and substrate control assays or the value for T_0 tests. However, when neuraminidase assays are performed with less well characterised materials, the results of separate enzyme and substrate control tests incubated for the same period as the test reaction mixture should be used for correction of assay results in case there is release of chromogenic material from the test substance itself during incubation of the test (e.g. see Section VIa).

Procedures for progress experiments to study the rate of NANA release. It would be convenient to be able to perform progress experiments in a bulk reaction mixture, taking samples at intervals and holding them at some stage before completion of the assay for NANA so that all results could be read in a single batch. In the standard neuraminidase assay the reaction is stopped by addition of Reagent 1 (periodic acid); the NANA assay proceeds immediately. Various attempts to hold the assay tubes at each stage during the NANA assay proved unsuccessful; consistent assay results were obtained only when all assay tubes were put through the uninterrupted assay for NANA in a single batch. The period between extracting the chromophore into Reagent 4 (acid butan-1-ol) and reading the assays was not critical; when assay tubes at this stage are held in the refrigerator or at room temperature for a few hours there may be a visible change in colour but the spectrophotometric reading (E_{549}) is unchanged.

Unsuccessful attempts were make to stop the neuraminidase action at the end of the test period of incubation, before the start of the assay for NANA. Assay tubes were put in a boiling water bath for 1.5 min and then held in the refrigerator for varying periods of time before assay for NANA; however, this did not completely inactivate the enzyme and also produced an unacceptable reduction in the final assay values. Other attempts were made to hold assay samples at 0°C for varying periods before the NANA assay. However, it proved impossible to prevent some enzyme activity when a series of unincubated reaction mixtures, prepared with pre-cooled reagents as for T_0 tests, were held at 0°C in an ice-water bath for graded periods up to 60 min before the start of the NANA assay. The results presented in table II/III show that even under these conditions there was a small amount of activity, related to the amount of enzyme present.

In further progress experiments, assays were always performed in separate tubes incubated for different periods of time; the timing of the start of each assay was so arranged that the test periods of incubation ended simultaneously and all tests were assayed for NANA in a single batch at the end of the experiment.

IId. Determination of conditions for optimal activity of C. perfringens neuraminidase

The effect of temperature on C. perfringens neuraminidase activity. Two experiments were performed in which standard assay reaction mixtures prepared with enzyme P9D3, diluted 1 in 3, and substrate FVII(6) were incubated for 60 min in a series of waterbaths held at different temperatures between 33°C and 60°C;

TABLE II/III

Effect of holding neuraminidase-assay reaction mixtures at 0°C for graded periods before the start of the assay for NANA

Time at 0°C before start of NANA assay[a] (min)	Spectrophotometric reading[b] with stated concentration of enzyme P9D2 (E_{549})	
	50%	100%
15	0.020	0.019
30	0.020	0.031
45	0.022	0.060
60	0.037	0.076

[a] Standard neuraminidase-assay reaction mixtures containing Clostridium perfringens enzyme preparation P9D2 and substrate FVII(5) in acetate buffer, pH 5.1 (see Materials and Methods) were prepared at intervals and held at 0°C in an ice-water bath before the start of the NANA assay.

[b] Test spectrophotometric readings (E_{549}) corrected by subtraction of the assay value for a control test (T_0 test) taken through the NANA assay immediately after addition of enzyme (average of triplicate assays).

one experiment covered the temperature range from 33°C to 50°C and the other extended the observations to 60°C. The data plotted in fig. II/6 show that similar curves were obtained where the two experiments overlapped. There was a steady increase in enzyme activity with increases in the temperature of incubation up to a maximum at c. 45°C. There was little change in activity as the temperature was further raised to 55°C, but a considerable decrease in activity in assays incubated at 60°C. The assay value for samples of NANA similarly treated over this range of temperatures remained relatively unchanged; thus the low assay value for neuraminidase at 60°C is not attributable to inactivation of NANA.

These data show that the activity of C. perfringens neuraminidase at 37°C might be increased by c. 35% by raising the temperature of the reaction mixture to 45°C. We have continued to use an incubation temperature of 37°C in standard assays; however, it should be appreciated that slight variations in the temperature of the waterbath used for incubation of reaction mixtures may contribute to differences in assay values in different batches of tests.

The effect of pH and buffer system on C. perfringens neuraminidase activity. Standard assay mixtures were prepared with enzyme preparation P9D2 and substrate FVII(5) but with buffers at different pH values (see Materials and Methods). An initial experiment confirmed that the assay for 10 µg pure NANA was unchanged when performed in acetate buffers at different pH values over the range 3.5-6.8. The results of neuraminidase assays in sodium acetate buffers over this pH range are shown in fig.

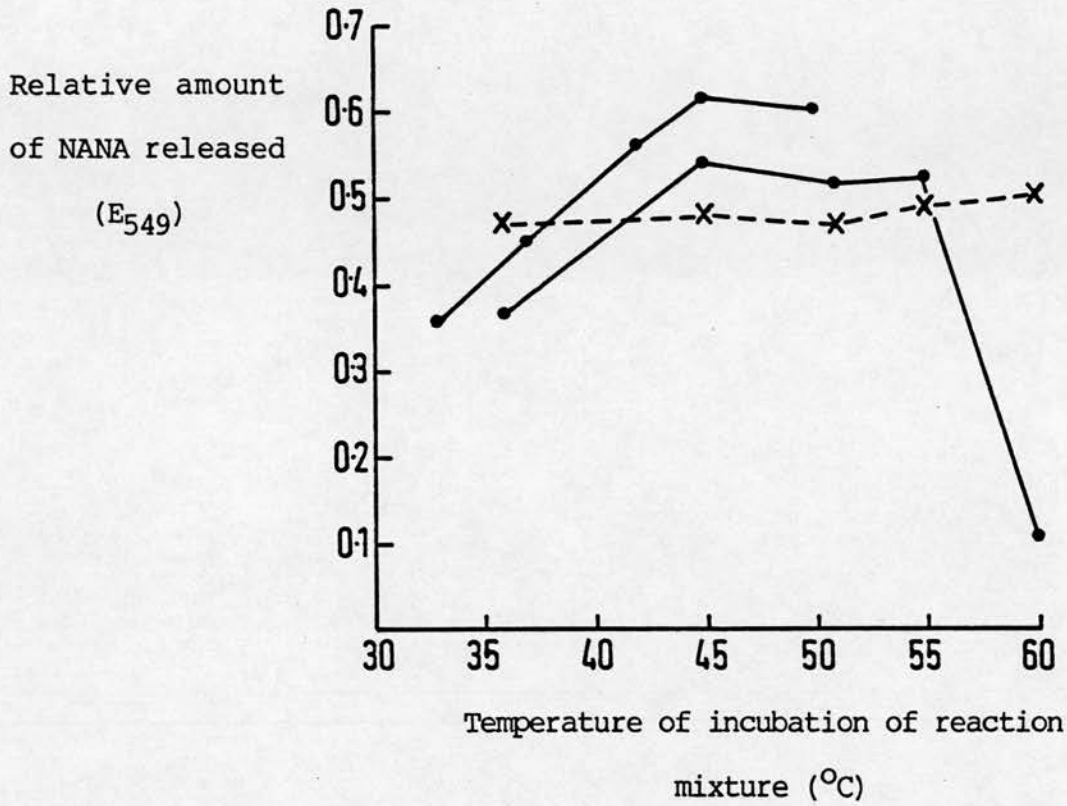


Fig. II/6 - Effect of temperature on activity of Clostridium perfringens neuraminidase. Activity plotted as the relative amount of NANA released by a standard amount of enzyme P9D3 incubated with substrate FVII(6) in acetate buffer, pH 5.1, for 60 min at different temperatures. The spectrophotometric values (E_{549}) for duplicate tests at each test temperature were corrected by subtraction of the sum of the values for separate enzyme and substrate control tests: ●—●, results of neuraminidase assays (two experiments); x--x, results of assays of pure NANA (10 µg) similarly incubated for 60 min at different temperatures.

II/7(a). The plot of uncorrected test results shows a major peak of activity at c. pH 4.6 and a subsidiary peak at c. pH 5.6; there was close agreement between the results for duplicate assays at each pH value tested. The results for single-tube substrate control assays are also plotted and these suggest that the main peak was actually at pH 4.7. The enzyme control readings were very low and are not plotted.

Fig. II/7(b) shows the results of a similar experiment when tris-maleate buffers were used. There was a single peak of activity at pH 5.7.

For these experiments, the initial pH of the reaction mixtures was measured directly in a parallel experiment (see Materials and Methods). Similar measurements were made after incubation of the reaction mixtures in the acetate buffers for 30 min at 37°C; in no case was there a deviation of more than 0.05 pH unit during the assay period. Direct readings of the initial and final pH of standard neuraminidase assays of dialysed and undialysed samples of culture filtrate P9 in acetate buffer, pH 5.1, showed that the initial pH of the standard reaction mixtures might be pH 5.30-5.45, but did not vary during incubation for 30 min at 37°C.

The effect of Ca^{2+} and EDTA on C. perfringens neuraminidase activity. Table II/IV compares the effect of varying the concentration of Ca^{2+} in assays of neuraminidase from C. perfringens and from Vibrio cholerae. When V. cholerae RDE was incubated with substrate FVII(7) for 15 min at 37°C in reaction mixtures containing different concentrations of Ca^{2+} or EDTA, it was

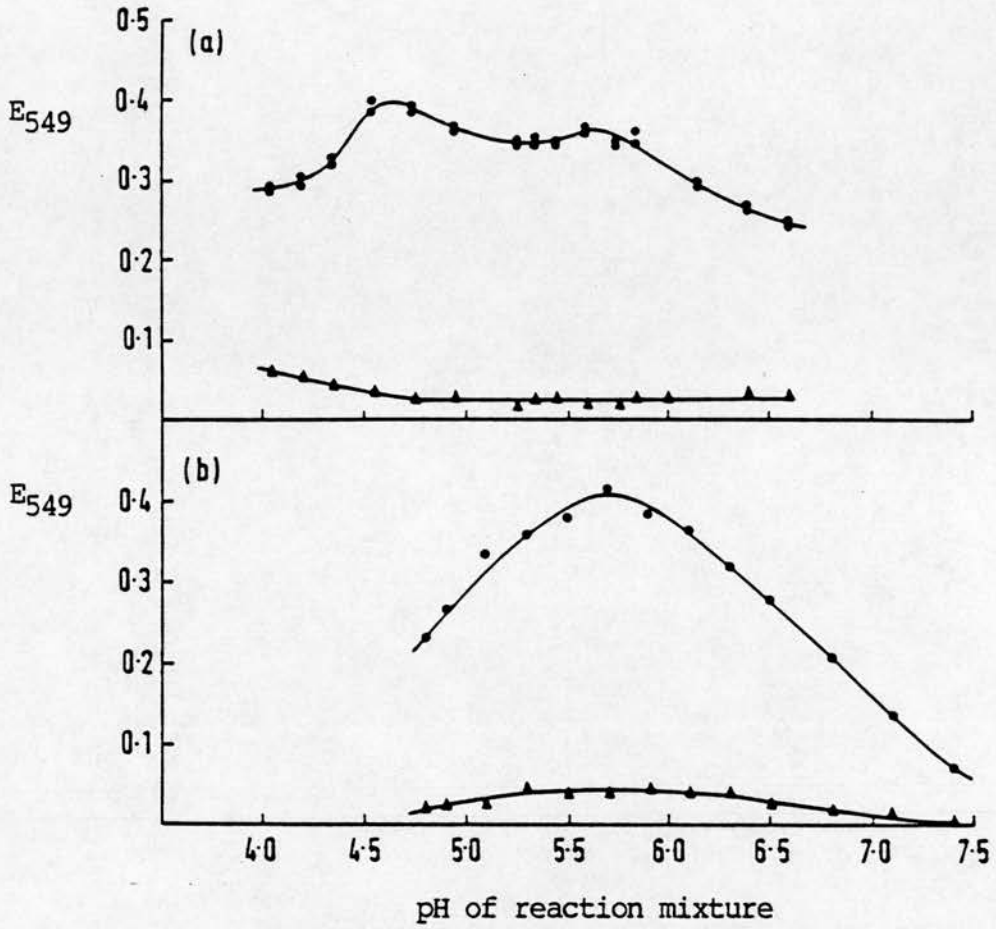


Fig. II/7 - Effect of pH and buffer system on the activity of Clostridium perfringens neuraminidase: (a) in sodium acetate buffers; (b) in tris-maleate buffers. Enzyme preparation P9D2 incubated with substrate FVII(5) at 37°C for 30 min. Activity plotted as absorbance (E_{549}) of the assay product for the reaction mixture: ●—●, test mixtures (uncorrected values); ▲—▲, substrate control tests.

TABLE II/IV

Effect of Ca^{2+} and EDTA on the assay of neuraminidase from Clostridium perfringens and Vibrio cholerae

Concentration of added Ca^{2+} or EDTA in reaction mixture[a]	Result of 15-min neuraminidase assay[b] on stated preparation (E_{549})	
	C. perfringens (P9D3)	V. cholerae (RDE)
3mM Ca^{2+}	0.348	0.576
1mM Ca^{2+}	0.315	0.436
None added	0.351	0.318
1mM EDTA	0.313	0.202
5mM EDTA	0.277	0.012

[a] See Materials and Methods.

[b] The spectrophotometric value (E_{549}) is an average result obtained from duplicate assay readings corrected by subtraction of the sum of the values for separate substrate and enzyme control tests. See Materials and Methods for tested enzyme preparations P9D3 and RDE.

confirmed that it was very calcium-dependent. The activity in our assay was almost doubled by the addition of 3mM Ca^{2+} and virtually abolished by 5mM EDTA. By contrast, when C. perfringens enzyme preparation P9D3 was assayed under the same conditions it showed little calcium dependence; the activity was not increased by addition of 3mM Ca^{2+} and 5mM EDTA reduced the assay value by c. 20% only. Ca^{2+} was not added to our assays as a routine.

IIe. Reproducibility of the neuraminidase assay

A series of dilutions of enzyme preparation P9D2 was made in acetate buffer, pH 5.1. Assay tubes containing 0.1 ml enzyme dilution mixed with 0.15 ml of the acetate buffer were prepared and stored at -20°C. On eight occasions over a period of ten weeks, tubes were brought to room temperature and the contents were assayed for neuraminidase activity by adding to each tube 0.25 ml of substrate FVII(5) and incubating at 37°C for 15 min. Time-zero (T_0) control tests were used for correcting the test results. All assays were performed in duplicate and average values were used in calculating the results plotted in fig. II/8. The means of the results obtained with graded concentrations of the enzyme preparation show the expected linear relationship. Reference tubes containing 10 µg pure NANA in 0.5 ml distilled water were also assayed on each occasion as a check on the reagents; the results fell in the range E_{549} 0.551-0.563 (mean 0.554).

The ranges of the results in this experiment give an indication of the reproducibility that was achieved with carefully standardised assays performed over a period of ten weeks during which fresh batches of assay reagents were made up as required.

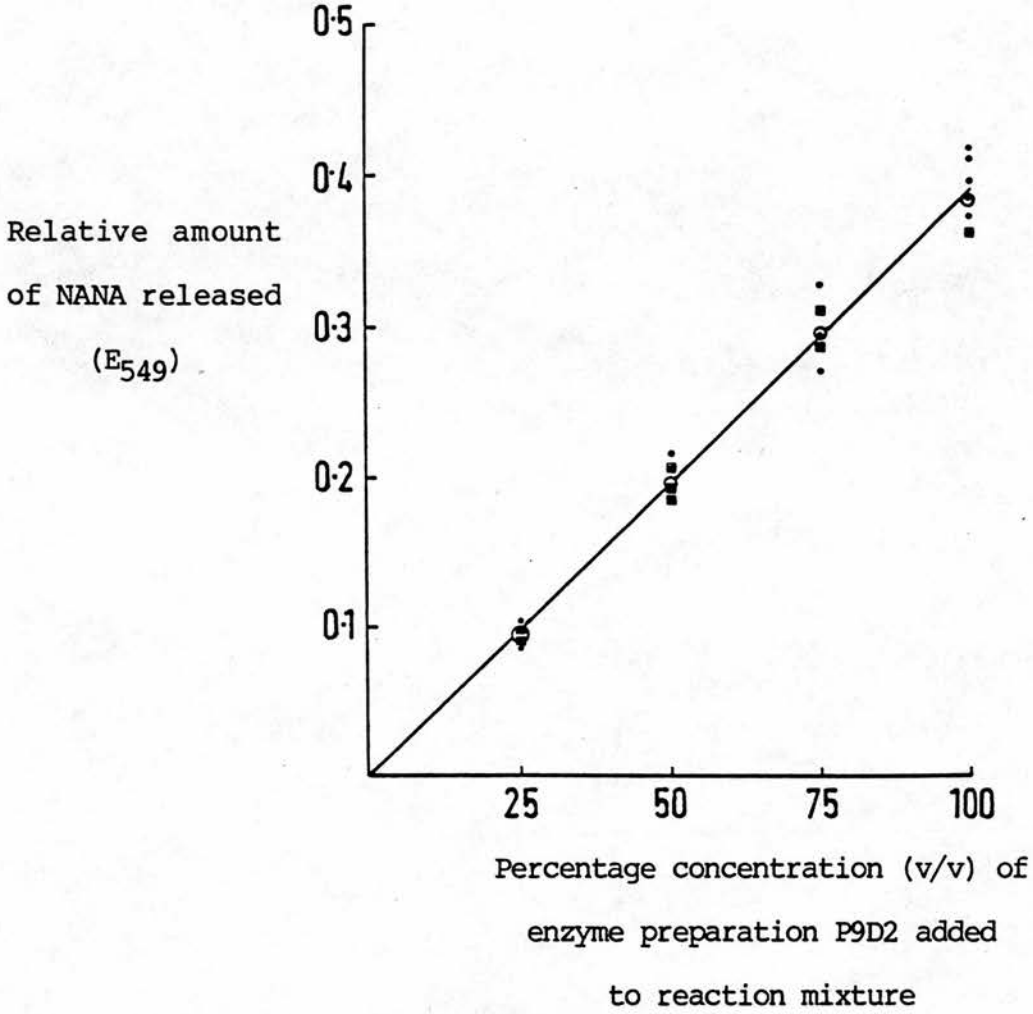


Fig. II/8 - Reproducibility of neuraminidase assay. Replicate 15-min assays performed on 8 occasions over a period of 10 weeks (see text for details). Test results (E_{549}) corrected by subtraction of the value for the corresponding T_o control test: ●, individual results; ■, two or more results; o, mean of 8 results.

However, over longer periods of time the value for assays of reference NANA samples (10 μg) proved more variable (see Section IIa) and assay values for standard neuraminidase preparations might also vary considerably. For accurate comparisons of neuraminidase activity in different preparations the tests should be performed simultaneously in a single batch of assays.

IIf. Neuraminidase activity of stock C. perfringens culture preparations

Table II/V gives the results of an experiment that compared the neuraminidase activities in the dialysed and undialysed preparations of the bulk culture filtrate (P9) of C. perfringens strain L2Ab used as stock enzyme preparations in these studies (see Materials and Methods). Interfering substances in the PPW5 broth gave rise to a high enzyme control value (E_{549} = 0.145) for the undialysed preparation P9; the dialysed preparations gave satisfactorily low control values. The value obtained on assay of 10 μg NANA in this experiment was used for conversion of the corrected spectrophotometric value for each test enzyme preparation into an estimate of the amount of NANA released during the 15-min incubation period; the observed neuraminidase activity was calculated from the rate of release of NANA (μmole/min) from the FVII substrate at 37°C in acetate buffer, pH 5.1 (1 unit neuraminidase releases 1 μmole NANA/min). The final column in table II/V shows these values adjusted for the slight increases in volume that occurred during dialysis of different batches of P9; it is apparent that there was little loss of activity during their preparation. It should, however, be appreciated that assay values

TABLE II/V

Results of 15-min neuraminidase assays with stock *Clostridium perfringens* strain L2Ab enzyme preparations

Test enzyme preparation[a]	Spectrophotometric reading (E_{549})			Neuraminidase activity	
	Test	Enzyme control	Corrected value[b]	Observed activity in tested preparation[c] (milliunits/ml)	Value adjusted for increase in volume during dialysis of samples[d] (milliunits/ml)
P9	0.702	0.145	0.505	23.2	23.2
P9D2	0.471	0.013	0.406	18.6	22.7
P9D3	0.432	0.008	0.372	17.1	18.6
P9D4	0.500	0.010	0.438	20.1	22.4

[a] See Materials and Methods.

[b] The corrected assay value is an average value obtained from duplicate test readings corrected by subtraction of the sum of the values for the FVII(8) substrate control (0.052) and the corresponding enzyme control.

[c] Observed neuraminidase activity calculated from rate of NANA release from substrate (μmole/min); reference sample of pure NANA (10 μg) gave a reading (E_{549}) of 0.470. (1 unit neuraminidase releases 1 μmole NANA/min from substrate.)

[d] Neuraminidase activity adjusted to allow for increase in volume during dialysis of original P9 preparation (see Materials and Methods).

for the undialysed preparation P9 are liable to give a slight underestimate of neuraminidase activity because interfering substances in the culture supernate must be assumed to have depressed the assay slightly; the values for dialysed preparations are more reliable (see Section IIa).

Purified C. perfringens neuraminidase was obtained commercially and used in comparisons with our stock enzyme preparations. Table II/VI gives the results of neuraminidase assays with Sigma neuraminidase and our preparations P9 and P9D2 incubated for 15 min with substrate FVII at 37°C in acetate buffer, pH 5.1. The Sigma neuraminidase does not interfere with the assay for NANA; the test concentration gave an enzyme control value of zero, and separate tests confirmed that it produced no depression of the assay value for 10 µg NANA and no reduction in reading during incubation with NANA at 37°C for 15 min.

The observed activities with the FVII substrate were calculated as in the previous experiment; there was good agreement between the values obtained for enzyme preparations P9 and P9D2 in the two experiments. The Sigma neuraminidase was stated by the manufacturers to have a potency of 1.1 unit/mg solid when tested with NAN-lactose, or 0.58 unit/mg when tested with bovine submaxillary mucin as substrate. The concentration of Sigma neuraminidase in the test solution (25 µg/ml) would thus have an activity of 27.5 milliunits/ml when tested with NAN-lactose, or 14.5 milliunits/ml with bovine submaxillary mucin. Comparison with the value for the observed activity of this preparation with the FVII substrate (24.8 milliunits/ml) suggests that the activities calculated for our enzyme preparations in tables II/V and II/VI

TABLE II/VI

Comparison of activity of stock neuraminidase preparations with reference preparation of purified Clostridium perfringens neuraminidase

Test enzyme preparation[a]	Spectrophotometric reading (E_{549})			Observed neuraminidase activity[d] (milliunits/ml)
	Test[b]	Enzyme control	Corrected[c] value	
Sigma	0.614	0.000	0.589	24.8
P9	0.729	0.174	0.530	22.3
P9D2	0.454	0.015	0.414	17.4

[a] Sigma = chromatographically purified neuraminidase (Sigma London Chemical Co. Ltd) at an initial concentration of 25 µg/ml (0.1 ml of this solution used in assay); P9 = undialysed supernate of culture of C. perfringens strain L2Ab in PPW5 broth; P9D2 = dialysed sample of P9.

[b] Average of duplicate test readings after incubation of reaction mixtures for 15 min.

[c] Test reading (E_{549}) corrected by subtraction of the sum of the values for the FVII(5) substrate control (0.025) and the corresponding enzyme control assay.

[d] See footnote to table II/V; reference sample of pure NANA (10 µg) gave a reading (E_{549}) of 0.513.

should be comparable to the values that would be obtained with NAN-lactose as substrate, and considerably higher than those with bovine submaxillary mucin.

Our stock enzyme preparations proved quite stable when stored undiluted at -20°C for several years, e.g. the several dialysed preparations of P9 were made at intervals over five years but continued to give comparable assay values throughout the present studies. The stability of dilutions of enzyme preparation P9D2 over a period of ten weeks at -20°C was demonstrated in fig. II/8. Solutions of purified Sigma neuraminidase proved much less stable at -20°C than our crude enzyme preparations. The Sigma neuraminidase used in the previous experiment was assayed immediately after the sample was dissolved and diluted for use in the test. Table II/VII compares the assay results for Sigma neuraminidase and enzyme preparation P9D3 in two experiments; aliquots of the test enzyme preparations, diluted for use in the assay as required, were held at -20°C for 11 days before performing the second batch of assays. The values obtained on assay of P9D3, and with reference samples of pure NANA, were very similar on the two occasions, but the value for this concentration of Sigma neuraminidase was reduced by c. 25% after storage for 11 days at -20°C.

TABLE II/VII

Comparison of activity of two neuraminidase preparations after storage at -20°C for 11 days

Test mixture[a]	Results of 15-min neuraminidase assays performed on two occasions[b] (E_{549})	
	Assay 1	Assay 2
Sigma + FVII	0.589	0.445
P9D3 + FVII	0.385	0.375
NANA (10 µg)	0.513	0.535

[a] Test mixtures were standard assay mixtures (0.5 ml) containing either 0.1 ml Sigma neuraminidase (25 µg/ml) or 0.1 ml undiluted enzyme preparation P9D3, mixed with substrate FVII(5) in acetate buffer, pH 5.1. Reference tubes contained 10 µg pure NANA in distilled water.

[b] Enzyme preparations held at -20°C for 11 days between Assay 1 and Assay 2. The spectrophotometric value (E_{549}) is an average result obtained from duplicate assay readings corrected by subtraction of the sum of the values for separate substrate and enzyme control tests; NANA assay values are for unincubated reference samples of NANA.

III. IN-VITRO PRODUCTION OF NEURAMINIDASE BY STRAINS OF CLOSTRIDIUM PERFRINGENS

IIIa. Production of neuraminidase by classical strains of C. perfringens type A

Time-course of neuraminidase production by C. perfringens strain L2Ab in batch-culture supernate. An apparatus was devised that allowed samples to be taken at intervals from a stirred anaerobic culture of C. perfringens strain L2Ab without opening the anaerobic jar or the incubator (see Materials and Methods). The time-course of bacterial growth and neuraminidase production was followed in 600 ml proteose peptone water (PPW5) broth culture maintained at 37°C for 72 h. The counting techniques used for constructing the growth curves shown in fig. III/1 are described in Materials and Methods. After a lag phase of c. 1 h, there was a steady logarithmic (log) rise in viable count until c. 6 h; the maximum count was reached at c. 8 h and the numbers remained stationary until 24 h. The results of total cell counts were very close to those of viable counts between 3 h (when the numbers became detectable by microscopy) and 24 h. Most of the cells were seen to occur as single cells and few formed clumps of more than two organisms even during the log phase of growth; the values plotted in fig. III/1 are total cell counts. The total count remained unchanged until 72 h, but the viable count began to fall after 24 h.

Separate viable counts were performed with pasteurised samples in order to detect spore formation. Up to 8 h there were no detectable spores (i.e. $<10^1$/ml); spores were rapidly formed

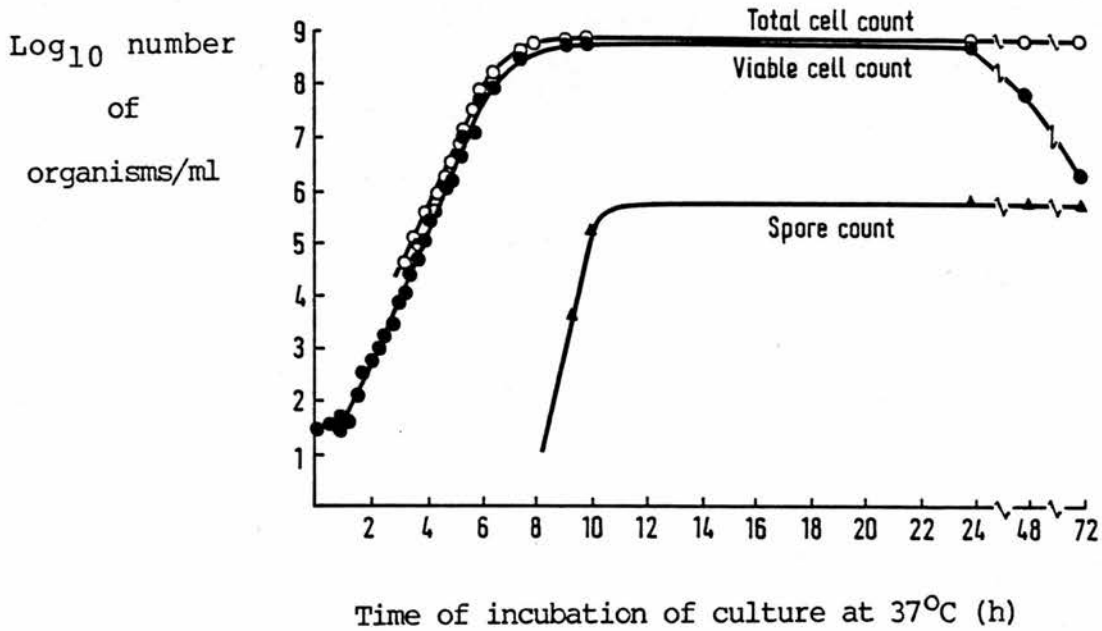


Fig. III/1 - Growth of Clostridium perfringens strain L2Ab in 600 ml PPW5 broth (see text for details): ●—●, viable count; o—o, total count; ▲—▲, spore count.

between 8 and 10 h but there was little further production by 24 h and no increase thereafter. Thus, spores were formed in late log phase, but represented only c. 0.1% of the total C. perfringens present even after 72 h incubation.

Samples of culture supernate were assayed for neuraminidase in standard tests incubated for 15 min at 37°C in acetate buffer, pH 5.1. High levels of activity were found in this experiment; samples were diluted appropriately in the assay buffer before the tests and the results plotted in fig. III/2 have been corrected accordingly. Further assays on the samples of culture supernate confirmed that there was no significant NAN-lyase activity in 24-h tests; thus the neuraminidase assay results are reliable even in the 48- and 72-h samples when the number of viable organisms was falling.

No neuraminidase was detected in 15-min assays of undiluted samples taken during the first 5 h of growth, but this was followed by a steady increase in enzyme activity in the culture supernate during late log phase between 6 and 10 h. Although both spores and neuraminidase were produced during late log phase, it is clear that considerable amounts of neuraminidase had already been produced before spores first became detectable at 9 h and that enzyme levels continued to rise after spore production had stopped. Enzyme production continued during early stationary phase but maximum levels had been reached by 24 h; the amount of neuraminidase activity in the culture supernate remained unchanged between 24 and 72 h. In general, cultures of clostridial species in later experiments were tested for neuraminidase production after incubation for 48 h.

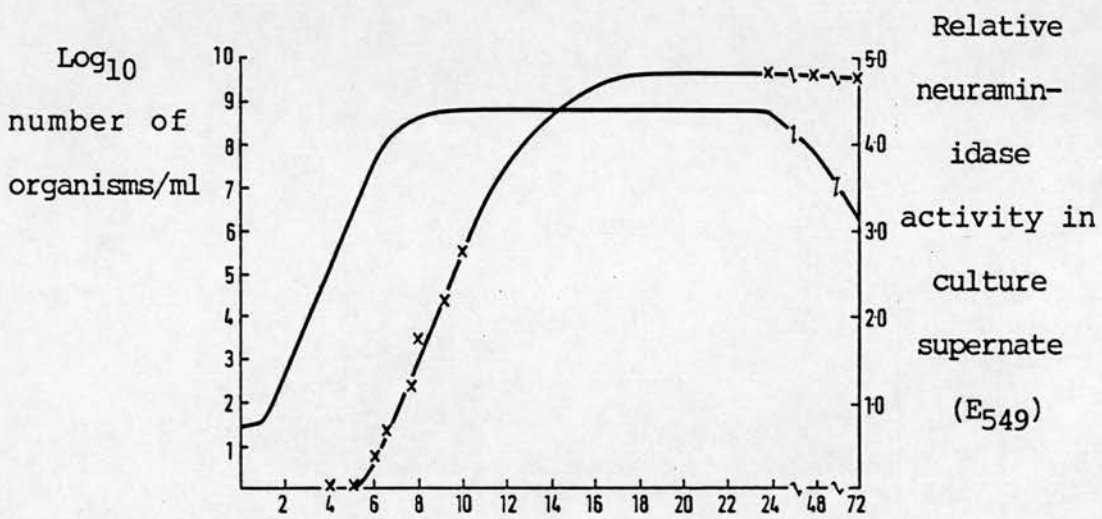


Fig. III/2 - Time-course of neuraminidase production by Clostridium perfringens strain L2Ab in 600 ml PPW5 broth. Neuraminidase activity (x—x) plotted as relative amount of NANA released (E_{549}) by samples of culture supernate in standard 15-min neuraminidase assays with substrate FVII(6). Samples diluted appropriately before assay; test results corrected by subtraction of the sum of the values for separate enzyme and substrate control tests and corrected for dilution factor. Solid line (—) = viable count, taken from fig. III/1.

Effect of temperature of incubation on production of neuraminidase by C. perfringens strain L2Ab. Twelve replicate 10-ml cultures of C. perfringens strain L2Ab were prepared in screw-capped bottles using a single batch of PPW5 medium and identical inocula (0.1 ml) from a 48-h PPW5 culture incubated anaerobically at 37°C. Triplicate cultures were incubated for 48 h in anaerobic jars placed on their sides in covered waterbaths accurately maintained at four different temperatures. Samples of the 12 culture supernates (800 g for 30 min at 4°C) were diluted 1 in 8 in acetate buffer, pH 5.1, before assay for neuraminidase; the results shown in fig. III/3 have been corrected accordingly. There was some variation in the assay values obtained for the triplicate cultures incubated at each temperature, but it is apparent that the incubation temperature was responsible for quite marked variations in the amount of neuraminidase produced. The maximum yield was obtained at 39°C and was approximately double that obtained at 35°C. It may be that minor differences in the temperature of incubation may contribute to variations in the amount of neuraminidase produced in apparently similar cultures on different occasions.

Variation in production of neuraminidase in replicate cultures of C. perfringens strain L2Ab. The amount of neuraminidase produced by a particular strain of C. perfringens under apparently similar cultural conditions was found to vary considerably on different occasions, although the factors responsible for this were not easily identified or controlled. An experiment was designed to show the extent of variation in neuraminidase production in

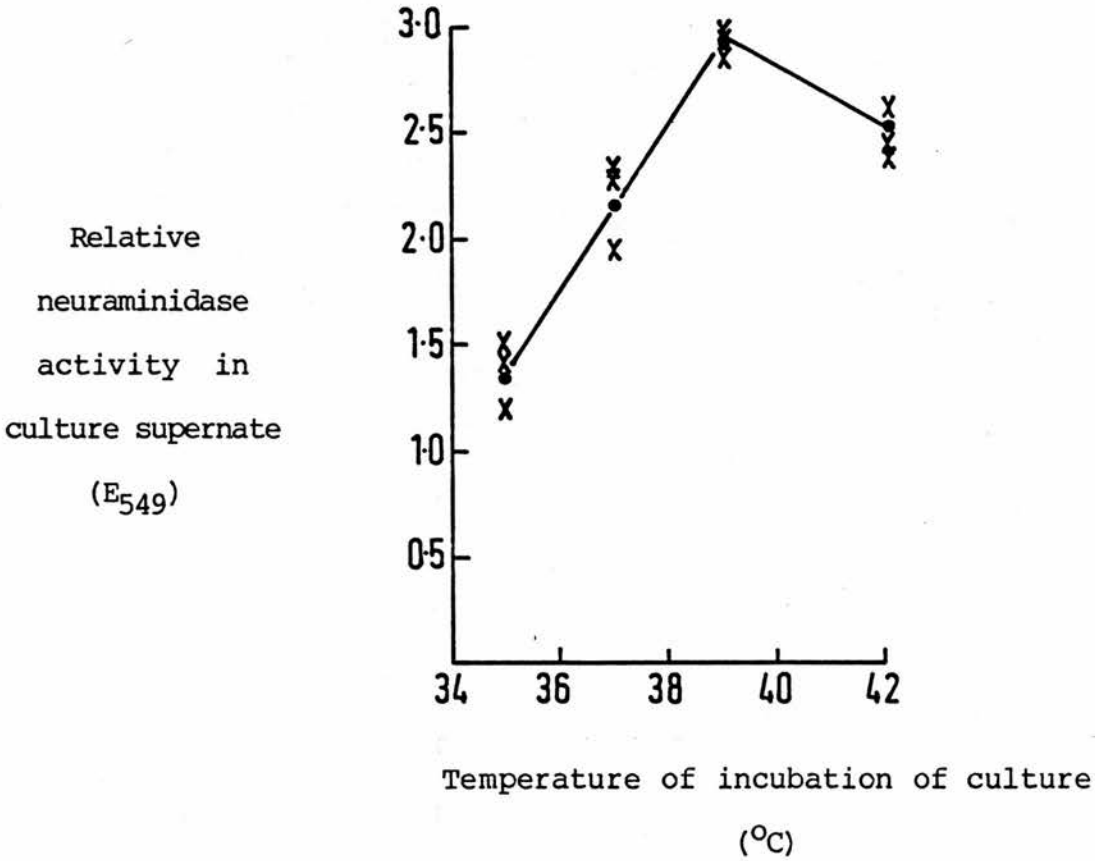


Fig. III/3 - Influence of incubation temperature on neuraminidase production by Clostridium perfringens strain L2Ab in PPW5 culture supernates. Triplicate cultures incubated at different temperatures (see text). Neuraminidase activity plotted as relative amount of NANA released (E_{549}) by samples of culture supernate in standard 15-min neuraminidase assays with substrate FVII(6). Samples diluted 1 in 8 before assay; test results corrected by subtraction of the sum of the values for separate enzyme and substrate control tests and corrected for dilution: x, individual assay results; •, mean of results for assays with triplicate cultures at each incubation temperature.

replicate cultures incubated simultaneously in the same anaerobic jar. Ten replicate 10-ml cultures of C. perfringens strain L2Ab were prepared, using a single batch of PPW5 medium and identical inocula (0.1 ml) from a 48-h PPW5 culture incubated anaerobically at 37°C. Ten replicate cultures of the same organism were similarly prepared in Todd-Hewitt broth (THB), with inocula from a 48-h THB culture. The 20 tubes were incubated together in an anaerobic jar at 37°C for 48 h. The centrifuged culture supernates (800 g for 1 h at 4°C) were tested in standard 15-min neuraminidase assays. Table III/I shows that there was a moderate degree of variation in neuraminidase production in each medium; it appears that the assay value may vary by c. 10% from the mean in such replicate cultures. In this experiment, about four times more neuraminidase was produced in PPW5 than in THB medium, but this pattern was not consistently found on other occasions (e.g. see table III/II).

Production of neuraminidase by C. perfringens strain L2Ab in serial cultures. The experiment shown in fig. III/4 was designed to demonstrate the variation in neuraminidase production that occurred when C. perfringens strain L2Ab was grown in serial 48-h cultures in two different media, and to compare enzyme production in the two media. After five serial cultures in PPW5 medium, five further serial cultures were made in nutrient broth (NB) medium, and these were followed by a further five serial cultures in the original medium. At the same time, a parallel series of 15 cultures was made, starting with five in NB, followed by five in PPW5, and reverting to NB medium for the final five cultures.

TABLE III/I

Neuraminidase production by Clostridium perfringens strain L2Ab in ten replicate cultures in each of two broth media

Results of 15-min neuraminidase assays on supernates of ten cultures in stated medium[a] (E_{549})	
PPW5	THB
2.848	0.717
2.984	0.732
3.176	0.753
3.256	0.771
3.304	0.780
3.344	0.786
3.376	0.795
3.400	0.855
3.408	0.858
3.512	0.894
Mean 3.261	0.794

[a] Results of standard 15-min assays with substrate FVII(6) for each replicate culture are the means of duplicate test readings (E_{549}), with no correction for enzyme or substrate control tests. Supernates of proteose peptone water (PPW5) cultures were diluted 1 in 8, and of Todd-Hewitt broth (THB) cultures 1 in 3, before assay; the results have been corrected accordingly.

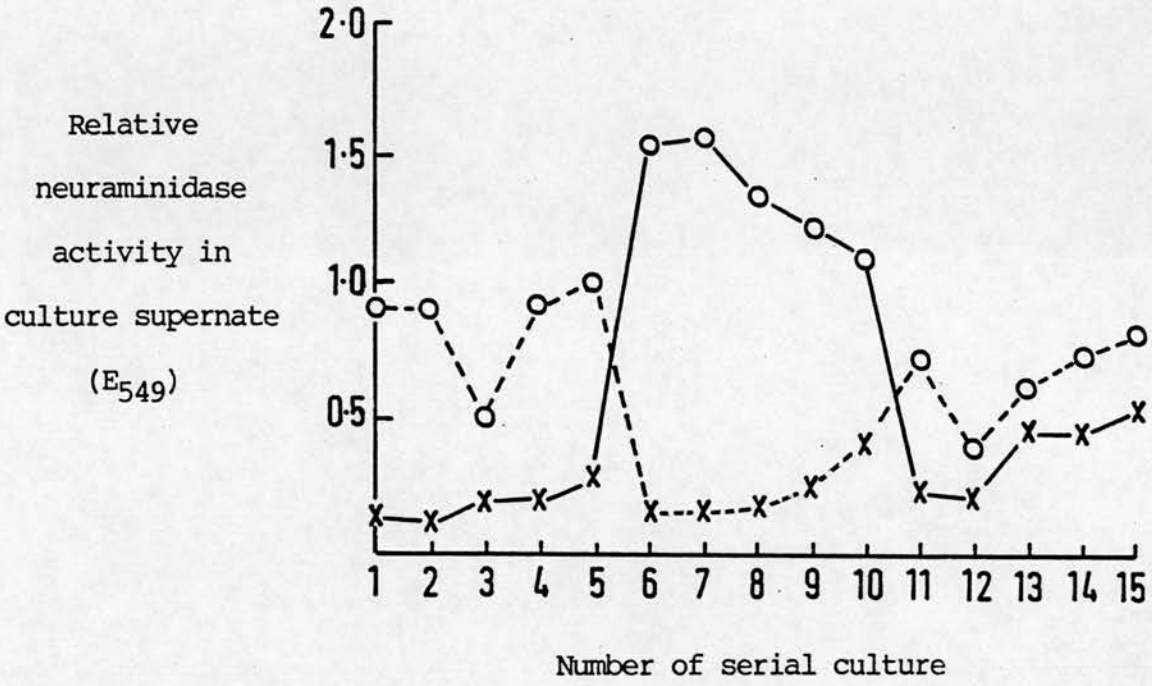


Fig. III/4 - Production of neuraminidase by Clostridium perfringens strain L2Ab in serial 48-h cultures in two broth media. Two experiments were performed, each with 15 serial cultures (see text); x = culture in nutrient broth (NB), o = culture in proteose peptone water (PPW5). Neuraminidase activity in the culture supernates is expressed as the result of standard 15-min neuraminidase assays (E_{549}) corrected by subtraction of the sum of the values for separate enzyme and substrate control tests.

A stock of tubes containing 10-ml amounts of culture medium was prepared from a single batch of each medium before the experiment; they were held at 4°C until pre-steamed just before inoculation. Initially, 0.1 ml of a 24-h anaerobic culture in cooked-meat broth (CMB) was used to inoculate the first tube in each series. Thereafter, each serial culture was incubated anaerobically at 37°C for 48 h and 0.1 ml of the 48-h culture was used as inoculum for the next culture in the series. Each serial culture was carefully checked for purity by subculture on blood agar (BA) plates incubated aerobically and anaerobically for 18 h at 37°C. Samples of each culture supernate (800 g for 1 h at 4°C) were held at -20°C until they were assayed for neuraminidase at the end of the experiment.

Fig. III/4 shows the results of 15-min assays for neuraminidase activity in the culture supernates of the two series of cultures; the assays were performed in two batches, one for each series of 15 cultures. There was considerable variation in the yield of neuraminidase on different occasions but it is apparent that there was usually a higher yield in PPW5 than in NB medium. Although there was some overlap between the range of assay values (E_{549}) found with PPW5 (0.39-1.02) and NB (0.11-0.55), the values in PPW5 broth were always higher than in the equivalent NB culture made at the same time, and there was a distinct step up or down in enzyme yield after each change of medium in both series of cultures. The degree of variation seen in these serial cultures is greater than that found in replicate cultures incubated in a single batch (table III/I). It seems probable that these variations are

partly attributable to minor differences in cultural conditions, e.g. temperature of incubation (see fig. III/3).

There might appear to be a tendency for serial cultures in NB medium to show a progressive increase in activity, but this is not borne out with the PPW5 cultures where the variation appears more random. In other similar series of cultures, with a variety of strains of C. perfringens grown in various culture media, no consistent pattern was found that would confirm sequential enhancement of enzyme production during serial culture; the initial cultures often produced high amounts of the enzyme and subsequent serial cultures might contain high or low levels, apparently at random (e.g. see fig. III/5 and table III/VII). However, as a routine, tests for clostridial neuraminidase were performed with the supernates of the second serial culture in the test medium.

Production of neuraminidase by classical strains of C. perfringens type A in various broth media. It proved difficult to choose a single culture medium that would consistently give maximum neuraminidase production with any strain of C. perfringens. Enzyme production in cultures of a single strain grown under apparently similar conditions but in different batches of medium and on different occasions over a period of months or years showed considerably greater variability than was seen with serial cultures using a single batch of medium over a period of 30 days (see fig. III/4). Thus, values for 15-min neuraminidase assays (E_{549}) with supernates of second serial cultures of C. perfringens strain L2Ab incubated at 37°C for 48 h in 10-ml volumes of PPW5 broth

varied from c. 0.5-3.5 on different occasions in these studies (c.f. values in figs III/3-5, tables III/I-III and VI).

It was not possible to identify differences between different batches of medium that might contribute to this. Preliminary experiments showed greater neuraminidase production in broth containing 5% proteose peptone than with lower concentrations. A number of unsuccessful attempts were made to identify inducing substances, e.g. by addition of FVII glycoprotein, NANA or proteose peptone to media in which little enzyme was produced. Although all these substances appeared on occasion to increase neuraminidase production in 48-h cultures, consistent results were not obtained. This was partly because the variability of neuraminidase production in the basic medium was too great to allow detection of small increases in enzyme production; however, in some repeat experiments the addition of these substances might actually correlate with decreased enzyme production.

Production of neuraminidase by four classical (haemolytic, heat-sensitive) strains of C. perfringens type A was compared in a range of broth media. Each strain was cultured for 48 h in each medium, and a second serial 48-h culture was made from each in the same medium. The results of neuraminidase assays on the supernates of the second serial cultures (table III/II) indicate that enzyme production is relatively poor in NB and generally good in PPW5 and THB media. These results reflect our general experience with many strains of C. perfringens and these two media were used in further studies of neuraminidase production by various clostridial species. Most clostridia grow well in either medium. Occasional cultures in THB medium were found to be unduly mucoid; this occurred rather

TABLE III/II

Production of neuraminidase by four classical strains of Clostridium perfringens type A grown in four broth media

Culture medium[a]	Result of 15-min neuraminidase assay[b] on supernate from second serial 48-h culture of stated strain (E_{549})			
	L2Ab	L3A	C1	032
NB	0.315	0.056	0.280	0.346
CMB	0.732	0.190	0.441	0.649
THB	1.320	0.656	0.692	0.900
PPW5	0.640	1.052	0.920	0.492

[a] NB = nutrient broth; CMB = cooked-meat broth; THB = Todd-Hewitt broth; PPW5 = proteose peptone water.

[b] The spectrophotometric value (E_{549}) is an average result obtained from duplicate assay readings corrected by subtraction of the sum of the values for separate substrate and enzyme control tests. Samples giving high assay values were diluted in acetate buffer, pH 5.1, before assay; the results have been corrected accordingly.

less frequently with PPW5 cultures. Although mucoid cultures were more difficult to handle, e.g. for centrifugation, there was no apparent effect on neuraminidase levels in the culture supernate. CMB cultures usually contained moderate amounts of enzyme but the presence of meat particles and debris makes these cultures relatively inconvenient and this medium was not used routinely in tests for neuraminidase production.

The results given in table III/III give an indication of the amount of growth that occurs in the different media in such cultures. Two serial 48-h cultures of strain L2Ab were prepared in each medium, using 10-ml volumes of medium and the same conditions for inoculation and incubation as for the previous experiment. Total and viable counts of organisms were made with the second serial culture in each medium (see Materials and Methods). The organism grew well in each culture medium; total counts did not differ markedly (1.1-6.7 x 10^8/ml) but there was a greater loss of viability in NB medium (c. 20-fold) than in the others (c. 2- to 3-fold).

The degree to which variations in neuraminidase production might correlate with variations in total or viable counts of organisms in 48-h broth cultures was not explored further, but attempts were made to ensure that all organisms tested for neuraminidase production were cultured in media that ensured good growth of the test strain. In later experiments, with other clostridial species (see Section IV), the amount of growth in cultures tested for neuraminidase production was recorded; all C. perfringens strains gave ++ or +++ growth in 48-h PPW5 cultures by this scale (see Materials and Methods).

TABLE III/III

Production of neuraminidase and amount of growth in 48-h cultures of Clostridium perfringens strain L2Ab in four broth media

Culture medium[a]	Number of organisms in second serial 48-h culture[b] (orgs/ml)		Result of 15-min neuraminidase assay on supernate from second serial 48-h culture[c] (E_{549})
	Total count	Viable count	
NB	1.1×10^8	7.4×10^6	0.427
CMB	1.1×10^8	7.0×10^7	0.391
THB	6.7×10^8	2.1×10^8	1.352
PPW5	2.7×10^8	9.3×10^7	0.722

[a] NB = nutrient broth; CMB = cooked-meat broth; THB = Todd-Hewitt broth; PPW5 = proteose peptone water.

[b] See Materials and Methods for counting techniques.

[c] See footnote to table III/II.

IIIb. Production of neuraminidase by food-poisoning strains of C. perfringens type A

Production of neuraminidase by food-poisoning C. perfringens of serotypes 1-24 and i-xviii. Reference food-poisoning strains of C. perfringens type A were obtained from Dr Betty Hobbs, Central Public Health Laboratory, Colindale (see Materials and Methods). Serotypes 1-24 are "typical food-poisoning strains" that are non-haemolytic and produce very heat-resistant spores. Serotypes i-xviii include both haemolytic and non-haemolytic strains; all produce relatively heat-sensitive spores. These strains were originally collected and classified by Dr Hobbs; more recently the two series of organisms have been amalgamated into a single series (serotypes 1-75) and revised serotype numbers have been allocated to strains i-xviii (see Discussion). For clarity, the original Hobbs' serotype numbers have been retained in this study.

All of the 41 reference strains grew well in PPW5 broth and the supernate of the second serial 48-h culture in this medium was tested for neuraminidase activity. The culture supernates were first tested in a screening assay incubated with substrate FVII(6) for 1 h; thereafter, positive supernates were assayed (at appropriate dilutions) by the standard 15-min assay procedure. The assay product of all positive tests was further examined with the scanning spectrophotometer to confirm that the absorption peak was at 549 nm. Table III/IV shows the relative amounts of enzyme produced by neuraminidase-positive strains (see Materials and Methods for grading of neuraminidase activity). Most of these strains produced large amounts of enzyme that gave high readings

TABLE III/IV

Enzyme production by neuraminidase-positive food-poisoning strains of Clostridium perfringens type A in supernates of PPW5 broth cultures

Serotype of test strain[a]	Relative amount of neuraminidase produced[b]
13	+++[c]
18	+++
21	++++
24	+[c]
i	+[c]
ii	+++
iii	+++
v	+++
vi	+++
vii	++++
ix	++++[c]
x	++++
xi	+++
xii	+++
xvi	+++
xvii	++++
xviii	++++
L2Ab	++++[c]

[a] Reference strains of different serotypes obtained from Dr Hobbs (see Materials and Methods).

[b] Supernate of second serial 48-h culture in PPW5 broth tested in assays with substrate FVII(6). Samples diluted in acetate buffer, pH 5.1, before assay where necessary. Test results corrected by subtraction of the sum of the values for separate enzyme and substrate control tests. See Materials and Methods for grading of neuraminidase activity.

[c] Positive results confirmed in repeat culture.

(E_{549} >0.1: +++ score) or very high readings (E_{549} >1.0: ++++ score) in 15-min assays; these are comparable to the amounts produced by the classical C. perfringens strain L2Ab in similar cultures. Two strains, Hobbs' types 24 and i, gave very low results in the 1-h screening assay (E_{549} <0.1). These supernates were reassayed in tests incubated for 4 and 24 h in order to demonstrate a progressive release of NANA during prolonged incubation; assays with both supernates were clearly positive in the 24-h tests (E_{549} >0.5: + score). A number of strains were retested in repeat cultures in order to check the consistency of our results; all were again clearly neuraminidase-positive. The Hobbs' type-13 and type-ix strains again produced high levels of enzyme and the type-24 strain again produced a rather small amount. The type-i strain, however, produced much larger amounts than before (+++ score); this suggested that it may be more variable than the type-24 strain.

Culture supernates that were negative in the 1-h screening assay were retested with 24-h incubation; all remained negative. It was confirmed that cultures of several strains (Hobbs' types 1-4, 11 and viii) remained negative in repeat cultures.

Table III/V summarises the results of the neuraminidase assays with both series of reference food-poisoning strains. Most of the typical non-haemolytic, heat-resistant C. perfringens strains were neuraminidase-negative, but there was good production of the enzyme by the reference strains of Hobbs' types 13, 18 and 21, and the strain of Hobbs' type 24 produced a small amount of activity. Thirteen of the 17 heat-sensitive food-poisoning strains produced neuraminidase but the remaining four strains were

TABLE III/V

Production of neuraminidase by heat-resistant and heat-sensitive reference food-poisoning strains of Clostridium perfringens type A in supernates of PPW5 broth cultures

Reference strains	Haemolytic effect on blood agar[a]	Serotype numbers of neuraminidase-positive strains[b]	Serotype numbers of neuraminidase-negative strains[c]
Hobbs' types 1-24 (heat-resistant)	NH	13, 18, 21, 24	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,14, 15, 16, 17, 19, 20, 22, 23
Hobbs' types i-xviii (heat-sensitive)	NH	i, ix	xiii, xiv, xv
	H	ii, iii, v, vi, vii, x, xi, xii, xvi, xvii, xviii	viii

[a] See Materials and Methods: H = haemolytic; NH = non-haemolytic.

[b] See table III/IV for relative amounts of neuraminidase produced.

[c] Tests incubated with substrate FVII(6) at 37°C for 24 h gave negative results.

completely negative. It appears that in general non-haemolytic strains do not produce neuraminidase whereas haemolytic strains do, though there are clear exceptions to this general correlation.

The same procedures were used to investigate neuraminidase production by a further four strains of Hobbs' serotype 13. Strains 029 and 153 were isolated from food-poisoning outbreaks in Edinburgh and strains 611 and 4621 were supplied by Dr Hobbs. The results presented in table III/VI show production of a moderate amount of neuraminidase by strain 611 and rather smaller amounts by the reference type-13 strain (NCTC10240) and by strains 029 and 153; nevertheless, activity was clearly demonstrable in tests incubated for 15 min (E_{549} >0.1). By contrast, there was no activity in the supernate of strain 4621 even when the tests were incubated for 24 h. When a sample of this supernate was incubated with NANA for 24 h under conditions equivalent to those of the neuraminidase assay, there was no significant reduction in the assay value for NANA; thus, the negative result in the 24-h neuraminidase assay was not due to destruction of NANA by NAN-lyase activity. It therefore appears that there may be variation in the ability to produce neuraminidase among strains of a single Hobbs' serotype of C. perfringens.

Tests for production of neuraminidase during serial cultures of food-poisoning strains of C. perfringens type A in PPW5 broth. Five serial 48-h cultures of the classical strain L2Ab and the neuraminidase-positive type-13 food-poisoning strains 029 and 153 were made in 10-ml volumes of PPW5 broth prepared from a single batch of medium at the start of the experiment. Fig. III/5 shows

TABLE III/VI

Production of neuraminidase by five strains of Clostridium perfringens of Hobbs' type 13 in supernates of PPW5 broth cultures

Strain number	Result of 15-min neuraminidase assay on supernate from second serial 48-h culture in PPW5 broth[a] (E_{549})
NCTC10240[b]	0.149
029	0.163
153	0.172
611	0.531
4621	0.000[c]
L2Ab	0.579

[a] See footnote to table III/II.

[b] Hobbs' type-13 reference strain.

[c] The negative result for the culture supernate of strain 4621 was confirmed when the period of incubation of the test was extended to 24 h.

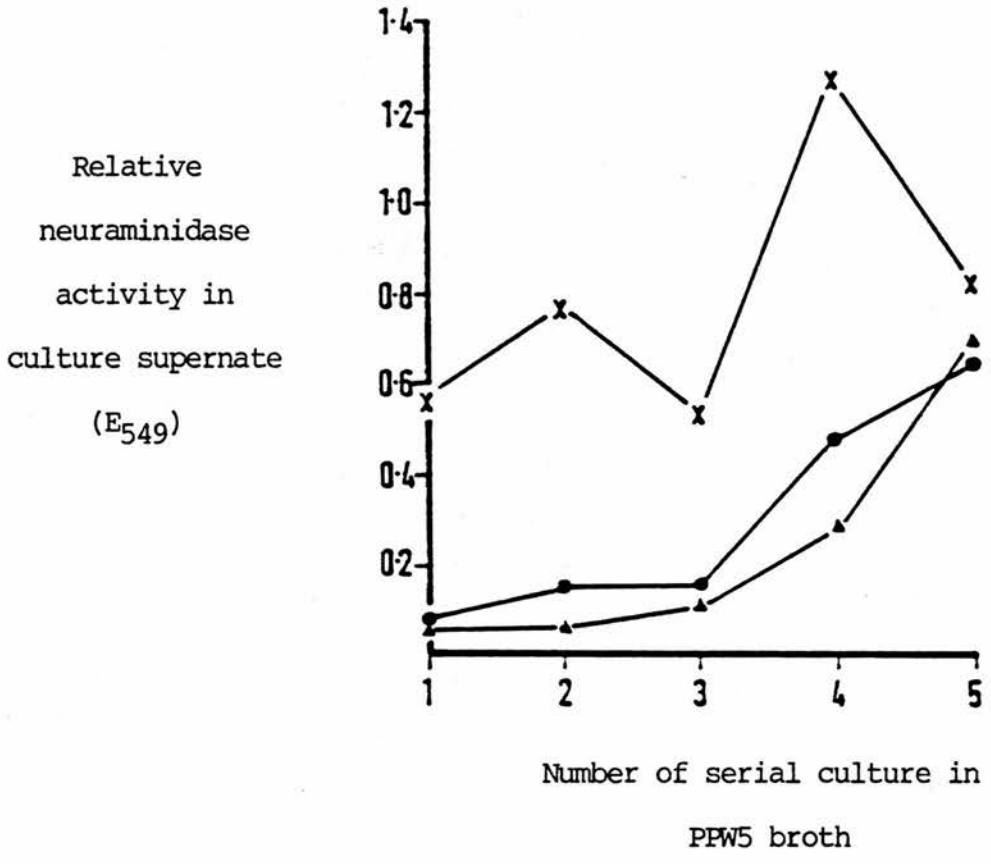


Fig. III/5 - Production of neuraminidase by Clostridium perfringens strains in serial 48-h cultures in PPW5 broth; x—x, strain L2Ab; ▲—▲, strain 153; ●—●, strain 029. Neuraminidase activity in the culture supernates is expressed as the result of standard 15-min assays (E_{549}) corrected by subtraction of the sum of the values for separate enzyme and substrate control tests. Strain L2Ab supernates were assayed at a dilution of 1 in 4 in acetate buffer, pH 5.1, and the values corrected accordingly; supernates of strains 153 and 029 were assayed undiluted.

the results of standard 15-min assays for neuraminidase activity in the culture supernates. Strain L2Ab produced moderate but variable yields of neuraminidase in all cultures. Initially, the two Edinburgh food-poisoning strains produced rather small amounts of enzyme but this increased during the serial cultures until by the fifth culture the activity was comparable with that produced by strain L2Ab. The presence of small amounts of activity in the first cultures in this series was confirmed by demonstrating that more NANA was released when the period of incubation of the assays was extended. In tests in which samples of supernate were incubated with a standard amount of NANA there was no significant breakdown of NANA. The values plotted in fig. III/5 therefore give a valid indication of the relative amounts of neuraminidase produced by the three strains in this experiment.

These results might suggest that sequential culture in PPW5 broth of strains 029 and 153 produced a gradually increased yield of neuraminidase. However, further experiments with organisms derived from the same freeze-dried stocks and grown under apparently identical conditions did not confirm this pattern; the initial cultures often produced high amounts of the enzyme and variations in yield could not be attributed to any particular variable in experimental conditions. The results of one such experiment, with strain 029, are given in table III/VII. Fifteen serial 48-h cultures were made in the same way as for the experiment with strain L2Ab shown in fig. III/4. After five serial cultures in PPW5 broth, five further cultures were made in NB medium and then another five in PPW5 broth; the supernates of the 15 serial cultures were tested in standard 15-min neuraminidase

TABLE III/VII

Production of neuraminidase by Clostridium perfringens strain 029 in serial 48-h cultures in two broth media

Number of serial culture	Culture medium[a]	Result of 15-min neuraminidase assay on culture supernate[b] (E_{549})
1	PPW5	1.668
2	PPW5	0.700
3	PPW5	0.680
4	PPW5	1.884
5	PPW5	5.184
6	NB	0.008[c]
7	NB	0.064[c]
8	NB	0.064[c]
9	NB	0.034[c]
10	NB	0.016[c]
11	PPW5	4.852
12	PPW5	3.012
13	PPW5	4.132
14	PPW5	4.116
15	PPW5	3.476

[a] PPW5 = proteose peptone water; NB = nutrient broth.

[b] See footnote to table III/II.

[c] Presence of small amounts of neuraminidase confirmed in assays incubated for 4 h.

assays. There were high enzyme yields in all cultures in PPW5 broth; the levels were again rather variable but there was good production in the initial cultures on both occasions. Small, but clearly demonstrable, amounts of activity were found in the 15-min assays with all the NB culture supernates.

Because of the apparent variation that may occur during serial cultures of neuraminidase-positive strains, four neuraminidase-negative C. perfringens strains were also tested in serial cultures. Five serial 48-h cultures of the reference strains of Hobbs' types 1-4 were made in PPW5 broth; these cultures were made as part of the experiment shown in fig. III/5, using the same batch of medium and incubating each serial culture of all strains in the same anaerobic jar. The results presented in table III/VIII show that there was no evidence of neuraminidase activity in any of the culture supernates of Hobbs' serotypes 1-4 when tested in 24-h assays. This was not due to destruction of NANA during the assay, as the results given in table III/IX show that there was no reduction in assay value when NANA was incubated with samples of the fifth serial culture supernates for 24 h under conditions equivalent to those of the neuraminidase assay. Thus, these typical food-poisoning strains remained neuraminidase-negative even when grown under conditions that simultaneously gave very good production of neuraminidase by the Edinburgh type-13 strains.

Assays for cell-associated neuraminidase. Five serial 48-h cultures of the neuraminidase-positive classical strain L2Ab and the neuraminidase-negative food-poisoning strain Hobbs' 2 were made successively at 39°C in 10-, 10-, 10-, 100- and 500-ml volumes of

TABLE III/VIII

Results of 24-h assays for neuraminidase in supernates of serial 48-h cultures of typical food-poisoning strains of Clostridium perfringens in PPW5 broth

Serotype of test strain	Number of serial culture	Spectrophotometric reading (E_{549})		
		Test	Enzyme control	Corrected value[a]
Hobbs' 1	1	0.193	0.169	-0.014
	2	0.192	0.171	-0.017
	3	0.196	0.172	-0.014
	4	0.194	0.165	-0.009
	5	0.191	0.162	-0.009
Hobbs' 2	1	0.182	0.159	-0.015
	2	0.181	0.158	-0.015
	3	0.179	0.159	-0.018
	4	0.180	0.156	-0.014
	5	0.179	0.163	-0.022
Hobbs' 3	1	0.188	0.155	+0.005
	2	0.183	0.162	-0.007
	3	0.177	0.158	-0.009
	4	0.165	0.148	-0.011
	5	0.167	0.147	-0.008
Hobbs' 4	1	0.169	0.151	-0.010
	2	0.168	0.146	-0.006
	3	0.172	0.156	-0.012
	4	0.164	0.147	-0.011
	5	0.166	0.147	-0.009

[a] The corrected assay value is an average value obtained from duplicate test readings corrected by subtraction of the sum of the values for the substrate and enzyme control tests. The tests were performed in two batches; the average of duplicate FVII(6) substrate control values was 0.038 for the assay of supernates of Hobbs' 1 and 2 serotypes and 0.028 for the assay of supernates of Hobbs' 3 and 4 serotypes.

TABLE III/IX

Effect of sterile PPW5 broth and of PPW5 culture supernates of typical food-poisoning strains of Clostridium perfringens on NANA determination in time-zero mixtures and in mixtures incubated at 37°C before assay

Test mixture[a]	Time of incubation at 37°C (h)	Spectrophotometric reading[b] (E_{549})		
		Test	Control	Corrected value[c]
NANA alone	0	0.466	...	0.466
	24	0.484	...	0.484
NANA + PPW5	0	0.500	0.121	0.379
	24	0.524	0.116	0.408
NANA + Hobbs' 1	0	0.514	0.151	0.363
	24	0.533	0.158	0.375
NANA + Hobbs' 2	0	0.490	0.149	0.341
	24	0.512	0.145	0.367
NANA + Hobbs' 3	0	0.494	0.155	0.339
	24	0.508	0.155	0.353
NANA + Hobbs' 4	0	0.509	0.149	0.360
	24	0.540	0.158	0.382

[a] Test mixtures contained 10 µg NANA in 0.25 ml distilled water, 0.1 ml test substance and 0.15 ml acetate buffer, pH 5.1. NANA alone = NANA + 0.25 ml buffer; PPW5 = sterile broth; Hobbs' 1-4 = supernate of fifth serial 48-h culture of respective strain in PPW5 broth (see table III/VIII).

[b] Average of duplicate assay readings; ... = not applicable.

[c] Test readings corrected by subtraction of the control value (0.1 ml test substance + 0.4 ml buffer).

PPW5 broth. Samples (100 ml) of culture supernates from the fifth serial cultures were concentrated c. 5-fold; the L2Ab culture was concentrated to 22.1% of its original volume, and the Hobbs' type-2 culture to 17.8%, after dialysis (see Materials and Methods). The washed cells from the 500 ml of the fifth serial cultures were resuspended in 20 ml saline and disrupted ultrasonically; the resultant suspension was then centrifuged free of cell debris and is referred to as cell extract. Table III/X presents the results of 15-min neuraminidase assays with substrate FVII(6) on the culture products of the strain L2Ab cultures. In this experiment the first culture in the series contained the greatest amount of enzyme but there were very high yields in the supernates of all five serial cultures; concentration of a sample of the fifth culture supernate produced a proportional increase in activity. The amount of neuraminidase in the successive washings fell rapidly to trace amounts, but there was release of a little further neuraminidase after ultrasonic treatment of the suspension of L2Ab cells; it should be appreciated that the enzyme in the cells from 500 ml culture was extracted into 20 ml saline and this concentration factor is reflected in the assay value of $E_{549} = 1.428$. By contrast, neuraminidase activity was not detected in any of the equivalent samples from the cultures of strain Hobbs' 2 even though the tests were incubated for 24 h.

Table III/XI shows the amount of NANA that was destroyed during incubation with samples of the fifth culture supernates for periods up to 24 h under conditions equivalent to those of the neuraminidase assay. The supernate of the fifth culture of strain L2Ab produced slight destruction of NANA after incubation for 24 h;

TABLE III/X

Neuraminidase activity in various culture products of Clostridium perfringens strain L2Ab grown in PPW5 broth

Product of serial culture[a]	Result of 15-min neuraminidase assay[b] (E_{549})
Supernate 1	5.480
Supernate 2	2.120
Supernate 3	3.404
Supernate 4	3.672
Supernate 5	3.088
Supernate 5 concentrate[c]	13.640
1st washing from bacillary deposit 5	0.433
2nd washing	0.032
3rd washing	0.021
Extract of sonicated washed deposit 5	1.428

[a] See Materials and Methods.

[b] See footnote to table III/II.

[c] Concentrated to 22.1% of the original volume.

TABLE III/XI

Effect of incubating NANA with culture supernates of Clostridium perfringens strains L2Ab and Hobbs' 2 for up to 24 h at 37°C

Reaction mixture[a]	Result of assay for NANA (E_{549})[b] after incubation of mixture for stated time					
	T_0	30 min	2 h	3 h	22 h	24 h
NANA alone	0.514	0.514	0.506	0.530	0.531	0.537
NANA+supernate of 5th serial culture of strain L2Ab	0.516	0.502	0.474	0.474	0.299	...
NANA+supernate of 5th serial culture of strain H2	0.503	0.500	0.494	0.511	0.511	0.500
NANA+concentrated supernate of 5th serial culture of strain H2	0.548	0.524	0.523	0.523	0.493	0.476

[a] Test mixtures contained 0.1 ml test substance and 10 µg NANA in 0.5 ml final volume (see footnote to table III/IX). For culture products, see text; H2 = Hobbs' type-2 strain.

[b] Spectrophotometric reading (E_{549}) of single-tube assays corrected by subtraction of the control value (0.1 ml test substance + 0.4 ml acetate buffer, pH 5.1); ... = not done.

this suggests that there is slight NAN-lyase activity in this culture supernate (although not enough to interfere appreciably with the 15-min neuraminidase assay). There was, however, no destruction of NANA by the supernate of the fifth culture of the strain of Hobbs' type 2 and even with this product concentrated 5-fold there was only a very slight reduction in the assay value for NANA after incubation for 24 h. This confirms that the results of the neuraminidase assays on culture supernates of the strain of Hobbs' type 2 are valid, i.e. that this strain produced no demonstrable extracellular neuraminidase.

Disruption of the washed cells of strain L2Ab and the strain of Hobbs' type 2 liberated NAN-lyase into the supernate. The results presented in table III/XII show that in each case the supernate from the third washing of the cells in saline produced no desctruction of NANA but that the supernate after disruption of the cells was able to destroy the test dose of NANA during incubation for 24 h. The amount of NAN-lyase activity in the cell extracts of the two strains appears to be comparable, as judged by the similar rate of NANA destruction during the first 3 h of the test.

Fig. III/6 shows the effect of the NAN-lyase in the L2Ab cell extract with neuraminidase assays incubated for periods up to 8 h. The amount of NANA released increased rapidly to reach a peak at 1 h and thereafter there was a progressive fall in assay value; thus the result of the 15-min neuraminidase assay on the L2Ab cell extract given in table III/X is liable to be an underestimate. Because the cell extract prepared from the Hobbs' type-2 strain contains a similar amount of NAN-lyase activity, the negative result in the 24-h neuraminidase assay on this sample might have

TABLE III/XII

Effect of incubating NANA with cell extracts of Clostridium perfringens strains L2Ab and Hobbs' 2 for up to 24 h at 37°C

Reaction mixture[a]	Result of assay for NANA (E_{549})[a] after incubation of mixture for stated time					
	T_0	30 min	1 h	2 h	3 h	24 h
NANA+3rd washing of strain L2Ab	0.477	0.467	0.481	0.474	0.470	0.461
NANA+cell extract of strain L2Ab	0.460	0.334	0.250	0.125	0.086	0.020
NANA+3rd washing of strain H2	0.485	0.502	0.494	0.487	0.489	0.492
NANA+cell extract of strain H2	0.480	0.372	0.300	0.163	0.113	0.008

[a] See footnotes to table III/XI.

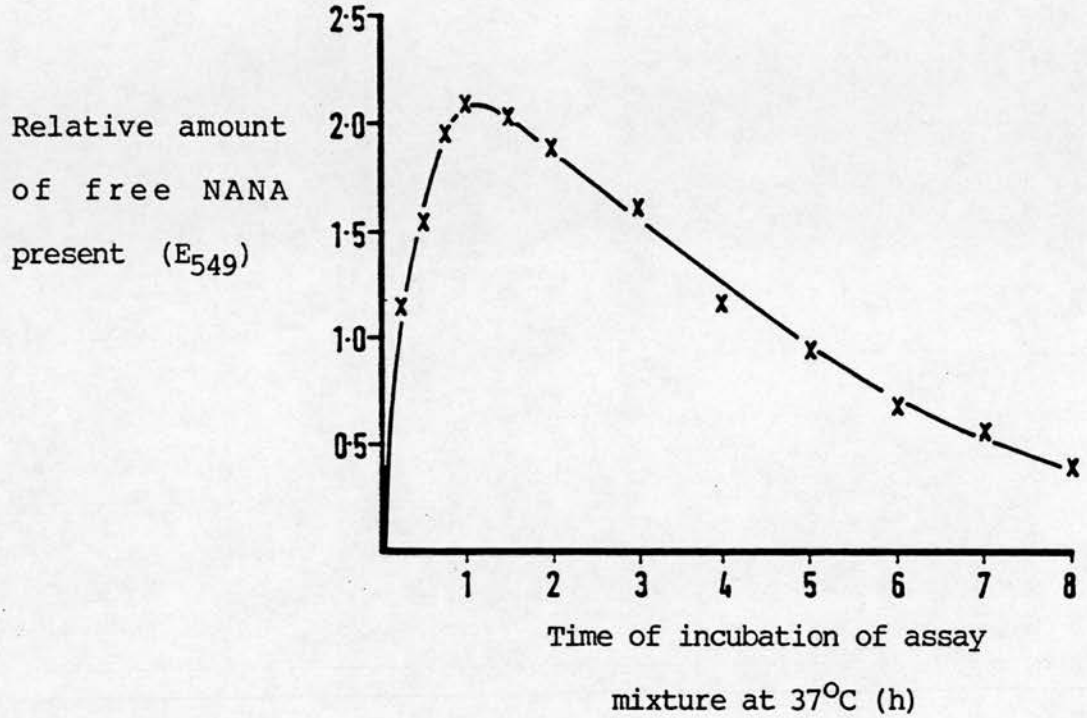


Fig. III/6 - Release and destruction of NANA during neuraminidase assays with undiluted cell extract from Clostridium perfringens strain L2Ab incubated with substrate FVII(6) for up to 8 h at 37°C. The relative amount of free NANA is plotted as the spectrophotometric reading (E_{549}) of single-tube assays corrected by subtraction of the value for the T_0 test. The assay product for each test was diluted 1 in 5 in reagent 4 (acid butan-1-ol) before reading and the results were corrected accordingly.

been due to subsequent destruction of the NANA released; however, further studies showed no detectable release of NANA even during the first 3 h of incubation of assay mixtures containing cell extract of this strain.

To give an indication of the amount of neuraminidase that we could expect to detect in the Hobbs' type-2 cell extract despite its NANA-destroying activity, dilutions of L2Ab cell extract were assayed in the presence and absence of the undiluted cell extract. The results are presented in fig. III/7. The assay values for neuraminidase were reduced by the Hobbs' type-2 cell extract in tests incubated for 2-3 h, but even 1% of the amount of neuraminidase released by ultrasonic treatment of strain L2Ab cells could clearly be detected in the presence of the cell extract prepared from the Hobbs' type-2 strain.

IIIc. Production of neuraminidase by various other strains of C. perfringens types A-E

Table III/XIII shows the results of standard tests for production of neuraminidase by various other strains of C. perfringens. All grew well (+++ growth) in PPW5 broth and the supernate of the second serial 48-h culture in PPW5 broth was tested for neuraminidase in screening assays incubated at 37°C for 60 min. The supernates were retested in 15-min assays and the assay product was examined in the scanning spectrophotometer to confirm that the absorption peak was at 549 nm.

The reference classical strain of C. perfringens type A, NCTC8237, and strains of C. perfringens of types B, C, D and E all

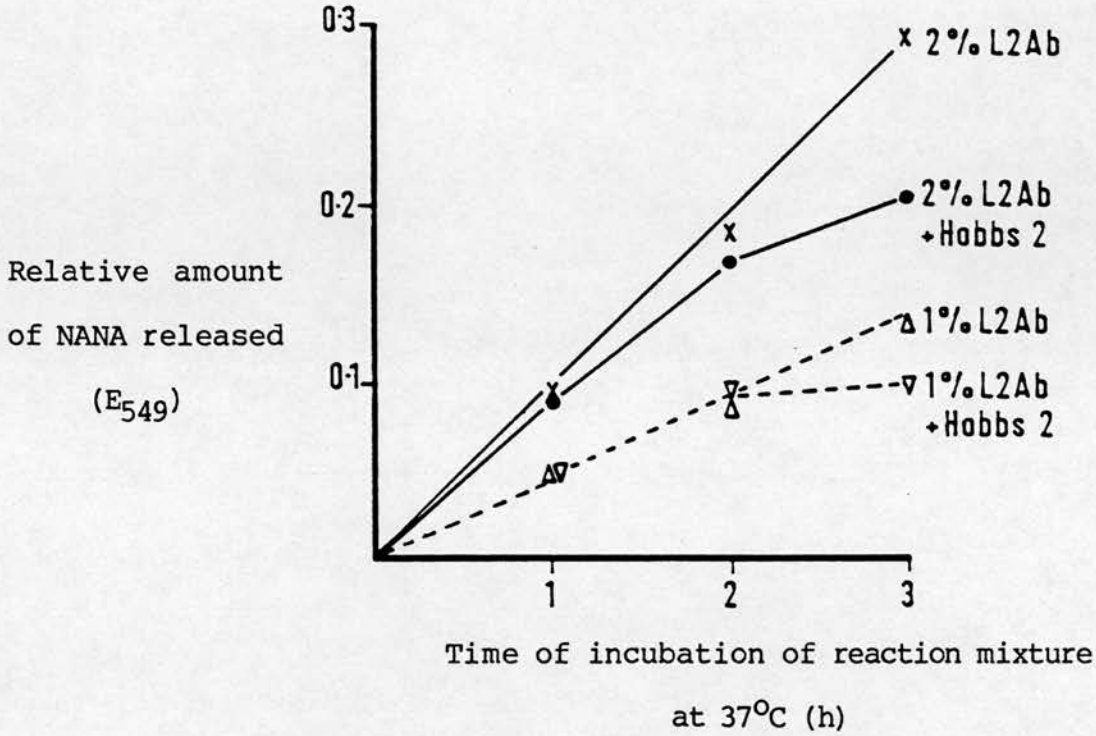


Fig. III/7 - Results of neuraminidase assays incubated for periods up to 3 h in the presence of cell extract of the Hobbs' type-2 strain of Clostridium perfringens. The test mixtures (final volume 0.6 ml) contained: 0.25 ml FVII(6) substrate; 0.15 ml acetate buffer, pH 5.1; 0.1 ml L2Ab cell extract diluted in buffer; and either 0.1 ml buffer or 0.1 ml undiluted cell extract of the Hobbs' type-2 strain. Test readings (E_{549}) corrected by subtraction of the value for the corresponding T_0 test: x—x, 2% (v/v) L2Ab cell extract + buffer; ●—●, 2% L2Ab cell extract + Hobbs'-2 cell extract; △—△, 1% L2Ab cell extract + buffer; ▽—▽, 1% L2Ab cell extract + Hobbs'-2 cell extract.

TABLE III/XIII

Production of neuraminidase by various strains of Clostridium perfringens of types A-E in supernates of PPW5 broth cultures

Strain number	Relative amount of neuraminidase[a] produced in stated medium	
	PPW5	THB
Type A		
NCTC8237	++++	...
NCTC11144	+++	++++
CW6	+++	+++
CW7	+++	+++
Type B		
NCTC3110	+++	...
Type C		
NCTC3180	++++	...
Type D		
L8	++	...
Type E		
NCTC8084	++++	...

[a] See footnote to table III/IV; ... = not tested.

produced good yields of neuraminidase. Three other type-A strains, NCTC11144, CW6 and CW7, were also shown to produce neuraminidase well in both PPW5 and THB media; these strains do not produce phospholipase-C (α-toxin) and are of interest in our later studies (see Section VII).

Mutant strains. A collection of mutant strains derived from the wild-type C. perfringens type A strain CN3870 (Rood & Wilkinson, 1975) was obtained from Drs Rood and Wilkinson and recharacterised in this laboratory. The phenotypic characters that these workers reported for these strains are summarised in table III/XIV. They selected mutant strains with reduced haemagglutinin production and noted that neuraminidase production was also reduced; in several cases this was linked with a marked reduction in sporulation frequency. Production of phospholipase-C was unchanged, apart from one strain (CM165) that showed an increase in activity. We were able to obtain three strains (CM156, 165 and 173) that showed a marked reduction in neuraminidase activity in their hands, but unfortunately their only neuraminidase-negative strain (CM171) was no longer available. They also selected revertant strains from a nalidixic-acid-resistant variant (CM198) of strain CM165; these strains (CM206, 209 and 218) all had increased sporulation frequency and were reported to have regained neuraminidase production to different degrees.

These strains were received by us as freeze-dried stock, with the caution that "the mutants revert very easily and must be cloned prior to each experiment" (J.I. Rood, personal communication, 1976). The results of our observations with these strains after

TABLE III/XIV

Properties of mutant strains of

Clostridium perfringens strain CN3870

Strain number[a]	Stated phenotypic characters[b]					Characters in this laboratory[c]		
	HA	SP	NM	PC	NA	NM	PC	NA
Wild-type strain								
CN3870	+	+	+	+	S	+	+	S
HA^- mutants								
CM156	-	-	±	+	S	+	+	S
CM165	-	-	±	++	S	+	+	S
CM171	-	-	-	+	S	...	...	...
CM173	-	+	±	+	S	+	+	S
NA-R mutant (165)								
CM198	-	-	±	++	R	-	-	R
SP^+ revertants								
CM206	±	+	±	+	R	+	+	R
CM209	-	+	±	+	R	+	+	R
CM218	+	±	+	++	R	+	+	R

[a] See text for description of mutant strains; strain CM171 no longer available.

[b] Data from Rood & Wilkinson (1975), table 2. HA = haemagglutinin; SP = sporulation frequency; NM = neuraminidase; PC = phospholipase-C: in each case + = activity of wild-type strain, ++ = increased activity, ± = reduced activity, - = no activity. NA = sensitivity to nalidixic acid (50 µg/ml); S = sensitive, R = resistant.

[c] Properties of strains determined in this laboratory (see text).

minimal subculture (but without any attempt to clone them) are also given in table III/XIV. The freeze-dried material was inoculated directly to CMB medium and to plates of BA and half-antitoxin EYA media for anaerobic incubation at 37°C for 24 h; a further BA plate was incubated aerobically. All cultures were confirmed to be anaerobic Gram-positive bacilli in pure culture. Nalidixic acid sensitivity was tested with a disk containing 35 µg of the drug added to the anaerobic BA plate; all strains produced the expected results. Strain CM198, however, proved not to be C. perfringens; the cells were smaller and of diphtheroid morphology, growth was slow and no phospholipase activity was detected even after incubation of EYA plates for 3 days. Further investigation showed it to be catalase-positive and it was tentatively identified as a Propionibacterium species. All of the other strains, including the nalidixic-acid-resistant derivatives of strain CM198, were C. perfringens, producing opalescence on EYA medium that was inhibited by the C. perfringens antitoxin.

The primary 24-h CMB culture of each strain was used to seed tubes of CMB, PPW5 and THB media, which were incubated anaerobically for 48 h at 37°C and assayed for neuraminidase. The organisms grew well in the test media and the supernates of all these cultures contained large amounts of enzyme activity (+++ or ++++ score, see Materials and Methods) in assays incubated for 1 h or 15 min; only the culture of the Propionibacterium strain was neuraminidase-negative. There was no detectable reduction in neuraminidase activity with any of the mutant strains. These strains were not further investigated in these studies; it was

felt that such unstable strains would be unlikely to be of value in tests for pathogenicity in animals.

IIId. Further characterisation of strains of *C. perfringens* type A

The strains of *C. perfringens* type A examined for neuraminidase production were further characterised for phospholipase-C (α-toxin) and haemolysin (θ-toxin) production. Phospholipase-C production was tested by culture on EYA plates (see Materials and Methods). The classical type-A strains L2Ab, L3A, C1, 032 and NCTC8237 were all phospholipase-positive, while strains NCTC11144, CW6 and CW7 were confirmed to be phospholipase-negative. All of the food-poisoning strains tested for neuraminidase production in tables III/V and VI were clearly shown to produce phospholipase well, apart from the strains of Hobbs' serotypes 14, viii and xvii, which produced much smaller zones of opacification in the medium and were classified as producing only low levels of phospholipase, and the type-18 strain, which was phospholipase-negative.

The strains were also tested for θ-toxin production by observing haemolysis on horse-blood agar (HBA) plates (see Materials and Methods). Horse red-blood cells are relatively resistant to the haemolytic action of phospholipase-C, which produces a fairly broad zone of partial haemolysis around the colonies of classical strains of *C. perfringens* type A on HBA plates; θ-toxin activity is seen as a smaller zone of complete ("beta") haemolysis around the colony. Thus non-haemolytic strains

(those that do not produce θ-toxin) may either show no haemolysis or only a zone of faint incomplete haemolysis attributable to phospholipase-C production (Willis, 1977, p. 131). The haemolytic reactions of the reference food-poisoning strains of C. perfringens type A recorded in table III/V indicate their production of θ-toxin; the other Hobbs' type-13 strains in table III/VI were also tested and found to be non-θ-toxin producing. The classical type-A strains L2Ab, L3A, C1, 032 and NCTC8237 were all haemolytic, as were the phospholipase-negative strains NCTC11144, CW6 and CW7. The properties of a number of C. perfringens type A strains that produced different combinations of these bacterial products and were selected for pathogenicity studies are tabulated later (see table VII/IX).

IV. IN-VITRO PRODUCTION OF NEURAMINIDASE BY OTHER SPECIES OF CLOSTRIDIUM

IVa. Examination of clostridial strains for neuraminidase production

Strains from a range of clostridial species were tested by standardised procedures developed from those used in studying neuraminidase production by strains of C. perfringens. The purity and identity of all strains were carefully checked as detailed in Materials and Methods. The supernate of the second serial 48-h culture was tested for neuraminidase as a routine. All strains were initially tested in PPW5 broth; most clostridia grew well in this medium. Most strains were also grown in THB medium, which occasionally gave better growth. When growth was poor in either medium (<+ score, see Materials and Methods), the cultures were repeated using a larger inoculum (1.0 ml) and prolonged incubation (96 h) for each serial culture. All strains that produced very little or no neuraminidase were tested in at least two media that supported adequate growth.

Culture supernates were initially tested in neuraminidase assays incubated for 60 min with substrate FVII(6) or FVII(7). When these were positive, the tests were repeated with 15-min incubation in order to give a more accurate indication of the amount of enzyme present. When the 60-min assay results were low or negative, the culture supernates were re-examined in tests incubated for 24 h. The relative amounts of neuraminidase produced by different strains were indicated on the same scoring system as was used for food-poisoning C. perfringens (see Materials and

Methods). The assay product from at least one test with each strain that gave a positive result was further examined in the scanning spectrophotometer to confirm that the peak absorbance was at 549 nm. Culture supernates that gave low-positive or negative results (score + or less) in 24-h neuraminidase assays were further tested to show that these results were not falsely low because of NAN-lyase production. Ca^{2+} was not added to the assays as a routine but a few neuraminidase-negative culture supernates were retested in the presence of Ca^{2+} in order to confirm that our inability to demonstrate enzyme activity was not due to lack of Ca^{2+} in the assay.

IVb. Production of neuraminidase by C. septicum, C. chauvoei, C. tertium and C. absonum

Table IV/I gives the data for production of neuraminidase by various strains and species of clostridia examined in this study; the results of tests with C. perfringens strain L2Ab are included for comparison. Strain L2Ab grew well in both PPW5 and THB media, producing large amounts of enzyme (++++ score) in the culture supernates. Cell-associated neuraminidase was also demonstrable in the cell extract of the PPW5 culture, although the enzyme is predominantly extracellular (see table III/X). The procedure used for making cell extracts in these experiments did not involve washing the packed cells to remove surface enzyme; it should also be appreciated that the cell extract was somewhat more concentrated than the original culture as the cells from 10 ml culture were used to produce 2 ml cell extract (see Materials and Methods).

TABLE IV/I

Production of neuraminidase by Clostridium septicum, C. chauvoei, C. tertium and C. absonum

Test organism and strain number	Growth in stated medium[a]		Relative amount of neuraminidase activity[b] in culture product		
			PPW5		THB
	PPW5	THB	Culture supernate	Cell extract	Culture supernate
C. perfringens					
L2Ab	+++	+++	++++	++++	++++
C. septicum					
NCTC547	++	+	++++	++++	+++
688/52	+	...	++++	...	...
1376/53	++	...	+++	...	...
2029/53	++	...	++++	...	...
CN3204	+++	...	+++	...	...
C. chauvoei					
NCTC8070	+[c]	-[c]	+	±	...
CC2	+[c]	-[c]	++	...	...
C. tertium					
NCTC541	+	++	+	...	±
CT1	+	++	+	±	±
C. absonum					
HA7103	+++	+++	+++	...	+++
HA7107	+++	+++	+++	...	+++
HA9103	++	+++	+++	...	+++

[a] See Materials and Methods for assessment of growth; ... not tested. PPW5 = proteose peptone water; THB = Todd-Hewitt broth.

[b] Culture supernate or cell extract from second serial culture in stated medium was tested. See Materials and Methods for grading of neuraminidase activity; ... = not tested.

[c] 96-h culture, 1-ml inoculum; other cultures incubated 48 h.

The strains of C. septicum grew adequately in PPW5 broth and all produced large amounts of enzyme in the culture supernate. Cell-associated neuraminidase was easily demonstrated in the PPW5 culture of strain NCTC547, and it was confirmed that the enzyme was also well produced in THB medium. Three strains of C. absonum also grew well in both test media and produced large amounts of enzyme in each.

The two strains of C. chauvoei tested did not grow well in PPW5 broth and they failed to grow in THB medium. Nevertheless, strain CC2 produced moderate amounts of neuraminidase in the PPW5 culture supernate, and neuraminidase production by strain NCTC8070 was clearly demonstrated when the incubation time of the assay was prolonged to 24 h; the enzyme was predominantly extracellular. The low values for neuraminidase activity in the culture supernate and the cell extract were not attributable to NAN-lyase activity. Both strains of C. tertium produced small amounts of neuraminidase; more enzyme was produced in PPW5 culture supernates although the organisms grew better in THB medium. The PPW5 culture supernates of C. chauvoei and C. tertium were further tested to confirm that their low neuraminidase activity was not increased by the addition of Ca^{2+} to the assay mixtures (see below, table IV/III).

IVc. Studies with C. sordelli and C. bifermentans

Table IV/II gives the data for neuraminidase production by 17 strains of these closely related species. Most of the C. sordelli strains grew well in PPW5 broth and all the culture supernates contained neuraminidase. The enzyme was predominantly extra-

TABLE IV/II

Production of neuraminidase by Clostridium sordelli and C. bifermentans

Test organism and strain number	Growth in stated medium[a]		Relative amount of neuraminidase activity[b] in culture product		
	PPW5	THB	PPW5		THB
			Culture supernate	Cell extract	Culture supernate
C. sordelli					
NCTC1340	++	+	++	...	+++
NCTC2914	+	+	+	...	++
NCTC6800	+	++	+	...	+
NCTC6801	+++	+	++	...	++
NCTC6927	++	++	+	...	++
NCTC6929	++	++	++	...	++
NCTC8780	+++	+	++++[c]	...	+++
1734	+++	+	+++[c]	...	+++
P3	+++	+	+++[c]	...	+++
CB2	+++	+	+++[c]	...	++
CB3	++	++	++[c]	±	+
CB4	++	++	+[c]	±	+++
C. bifermentans					
NCTC506	+++	++	-[c]	-	-
NCTC1341	++	++	-	...	-
NCTC6928	++	+++	-	...	-
B4	+++	+++	-[c]	-	-
1617	+++	+++	-[c]	-	-

[a] Cultures incubated for 48 h: see Materials and Methods for assessment of growth; ... = not tested. PPW5 = proteose peptone water; THB = Todd-Hewitt broth.

[b] See footnote to table IV/I.

[c] Result confirmed in repeat culture.

cellular in two strains whose cell extracts were also examined. Although several strains grew rather poorly in THB medium, enzyme activity was also clearly demonstrated in these supernates. By contrast, the five strains of C. bifermentans could not be shown to produce the enzyme in culture supernates of either medium and no cell-associated activity was demonstrated in 24-h assays with the cell extracts of three strains grown in PPW5 broth. These negative results were shown not to be due to NAN-lyase activity.

Enzyme control values used for correcting neuraminidase assays with THB culture supernates were low (E_{549} c. 0.06) and there was no difficulty in demonstrating the absorption peak at 549 nm of the chromophore formed in these assays. However, the PPW5 culture supernates often gave high enzyme control values (E_{549} = 0.2-0.3) due to the formation of a chromophore with peak absorption at c. 530 nm (see Section IIa) and this tended to obscure the presence of small peaks attributable to neuraminidase action. In this situation it was sometimes necessary to use a time-zero (T_0) control test as reference sample for spectrophotometry in order to confirm that a low corrected assay value was indeed due to formation of a product with peak absorption at 549 nm.

For example, detailed investigations were made with the PPW5 culture supernate of C. sordelli strain CB4 which contained rather small amounts of neuraminidase (score +). Standard assay mixtures were incubated at 37°C for 2 h. When the assay products were examined in the scanning spectrophotometer with reagent blank in the reference cell, both the test and the enzyme control contained a chromogen that gave a very high absorption peak at c. 530 nm. The shoulder of this curve gave a reading at 549 nm; the test

value (E_{549}) was 0.40 and the enzyme control value was 0.24; the substrate control value was very low (0.02). The corrected assay value (0.14) should indicate the release of a small amount of NANA, but fig. IV/1 shows that the absorption peak appeared to be at c. 530 nm when these products were compared. The explanation for this artefact is not clear but when the assay product of the T_0 test was used in the reference cell the presence of a small peak at 549 nm (c. 0.1) was clearly shown. It was confirmed that this was attributable to NANA release by extending the period of incubation and demonstrating a progressive rise in the peak at 549 nm, to c. 0.9 after 24 h. The distortion of the NANA peak was seen only with samples that contained very small amounts of NANA and that had rather high enzyme control values (E_{549}); the problem was avoided by using T_0 controls in confirmatory tests. Thus although equivocal results were initially obtained in standard 24-h assays with a few PPW5 culture supernates of C. bifermentans strains, comparison with T_0 control tests confirmed that there was no NANA release during incubation of assay mixtures for 24 h.

In order to confirm that low-positive or negative neuraminidase assay results were not due to lack of Ca^{2+} in the assay mixtures, the culture supernates of various clostridial strains were retested in the presence of added Ca^{2+} (table IV/III). The tests were performed with and without added 3mM Ca^{2+} in the assay mixtures, as was done previously with C. perfringens strain L2Ab (see table II/IV); in this case enzyme control tests were not included and uncorrected assay results were compared. Culture supernates with strong neuraminidase activity were diluted before testing in 15-min assays and the time of incubation of assays of

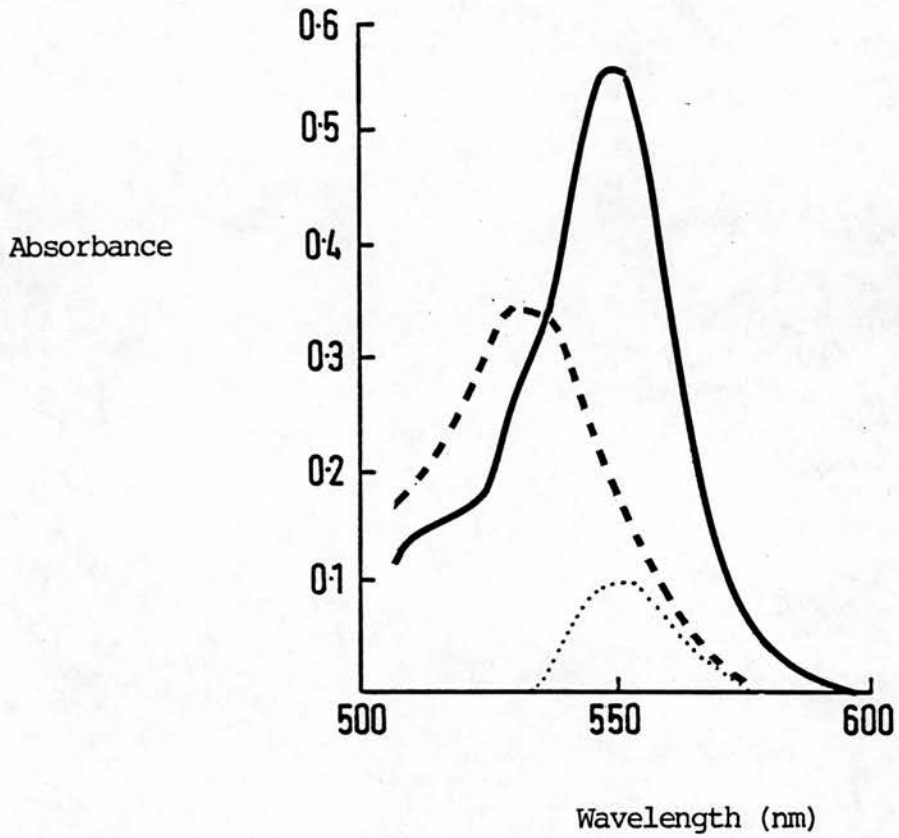


Fig. IV/1 - Comparison of T_0 control test and enzyme control test as reference preparation in determining the absorption peak of the neuraminidase-assay product with a PPW5 culture of Clostridium sordelli strain CB4. The PPW5 culture supernate (see table IV/II) was incubated in the assay mixture for 2 h at 37°C with substrate FVII(7). Absorbance curves read in the scanning spectrophotometer with the control assay product as reference preparation: ---, enzyme control test incubated for 2 h with no substrate; ..., unincubated T_0 control test (see Materials and Methods). The solid line indicates the curve obtained with assay of 10 µg of pure NANA read with reagent blank in the reference cell.

TABLE IV/III

Effect of Ca^{2+} on neuraminidase assays with culture supernates of various clostridia

Test organism and strain number[a]	Time of incubation of test mixture[b]	Result of neuraminidase assay[c] (E_{549})	
		With no added Ca^{2+}	With 3mM Ca^{2+}
C. perfringens			
L2Ab	15 min	0.334	0.327
C. septicum			
NCTC547	15 min	0.216	0.228
C. chauvoei			
NCTC8070	24 h	0.317	0.301
CC2	15 min	0.545	0.555
C. tertium			
NCTC541	60 min	0.324	0.335
CT1	60 min	0.311	0.314
C. sordelli			
NCTC8780	15 min	0.715	0.739
CB3	60 min	0.352	0.361
V. cholerae			
RDE	15 min	0.370	0.755

[a] Supernate of second serial culture in PPW5 broth tested (see tables IV/I and IV/II). For *Vibrio cholerae* RDE see Materials and Methods.

[b] Tests incubated at 37°C for the stated period before assay for NANA. Test material assayed undilute except *C. perfringens*, diluted 1 in 16, and *C. septicum*, diluted 1 in 8, in the acetate buffer, pH 5.1, before addition to the test mixture.

[c] The spectrophotometric value (E_{549}) is the uncorrected value for standard neuraminidase-assay mixtures with or without added Ca^{2+} (see Materials and Methods). Average of values for duplicate tests.

supernates with weak activity was prolonged in order to give assay results (E_{549}) in the range 0.2-0.8. The calcium dependence of Vibrio cholerae neuraminidase was clearly demonstrated but none of the clostridial enzymes showed any increase in activity. Further detailed tests were performed with the PPW5 and THB culture supernates of C. bifermentans strains NCTC506, NCTC1341, NCTC6928 and B4 that could not be shown to contain neuraminidase in standard assays (see table IV/II). The 24-h assays remained negative in the presence of added 3mM Ca^{2+}.

The identity of each of the strains of C. sordelli and C. bifermentans was carefully rechecked by the biochemical criteria shown in table IV/IV. All strains classified as C. sordelli were positive in tests for urease production and growth inhibition by mannose while the strains classified as C. bifermentans were all negative; this correlated exactly with our findings for neuraminidase production. All strains of C. sordelli failed to ferment mannose and sorbitol; most strains of C. bifermentans did ferment these substances but two gave inconsistent results in these tests. It appears that these fermentation tests may be less reliable in discriminating between the two species.

IVd. Studies with C. novyi and other neuraminidase-negative clostridia

No neuraminidase production was demonstrable in cultures of 10 strains of C. novyi types A-D. Table IV/V shows that some strains grew poorly in the test media, although the PPW5 broth was supplemented with cysteine and dithiothreitol (PPW5S) and a large inoculum and prolonged incubation time were used. No neuraminidase

TABLE IV/IV

Biochemical characterisation of strains of Clostridium sordelli and C. bifermentans

Test organism and strain number	Fermentation[a] of		Growth inhibition by mannose[a]	Urease production[a]	Neuraminidase production[b]
	Mannose	Sorbitol			
C. sordelli					
NCTC1340	-	-	+	+	+
NCTC2914	-	-	+	+	+
NCTC6800	-	-	+	+	+
NCTC6801	-	-	+	+	+
NCTC6927	-	-	+	+	+
NCTC6929	-	-	+	+	+
NCTC8780	-	-	+	+	+
1734	-	-	+	+	+
P3	-	-	+	+	+
CB2	-	-	+	+	+
CB3	-	-	+	+	+
CB4	-	-	+	+	+
C bifermentans					
NCTC506	+	+	-	-	-
NCTC1341	-	-	-	-	-
NCTC6928	+	-	-	-	-
B4	+	+	-	-	-
1617	+	+	-	-	-

[a] See Materials and Methods for tests and methods of reading results.

[b] Results from table IV/II.

TABLE IV/V

Growth of Clostridium novyi species obtained in three broth media

Test organism and strain number	Growth in stated medium[a]		
	PPW5S	THB	CMB
Type A			
NCTC538	+	++	+
NCTC6737	+	++	...
GR2A	+	++	...
GR4A	+	++	...
Type B			
GR1B	±[b]	+[b]	+
Type C			
NCTC9746	+[b]	+[b]	...
NCTC9747	+[b]	+[b]	++
Type D			
NCTC8145	+	++	...
NCTC8350	++	++	++
NCTC9692	++	++	...

[a] See Materials and Methods for assessment of growth; ... = not tested. PPW5S = proteose peptone water supplemented with cysteine and dithiothreitol; THB = Todd-Hewitt broth; CMB = cooked-meat broth.

[b] 96-h culture, 1-ml inoculum; other cultures incubated 48 h.

activity could be shown in culture supernates in PPW5S or THB media even in assays incubated for 24 h. Culture supernates and cell extracts of four strains grown in CMB medium were also neuraminidase-negative. No significant NANA destruction was found with the majority of these culture products; a few produced a small reduction in the assay value (E_{549}) for 10 μg NANA after incubation for 24 h, but in no instance was this greater than 0.15 and this would not have obscured significant neuraminidase activity. Tests similar to those with C. bifermentans (see Section IVc) were done to confirm that the addition of Ca^{2+} did not affect these negative results. The CMB culture supernates and cell extracts shown in table IV/V were reassayed in 24-h neuraminidase assays with and without 3mM Ca^{2+} but all remained negative.

Tables IV/VI and IV/VII show the other strains of clostridia that did not produce neuraminidase; no activity was demonstrable in 24-h assays in any of the culture supernates. A few strains that grew poorly in one of the test media showed better growth in the other. None of the culture supernates contained significant NANA-destroying activity.

TABLE IV/VI

Growth of Clostridium tetani, C. botulinum and C. difficile strains obtained in two broth media

Test organism and strain number	Growth in stated medium[a]	
	PPW5	THB
C. tetani		
NCTC279	++	++
NCTC540	+++	+
NCTC5404	++	++
NCTC5405	++	+
NCTC5413	++	+
NCTC9569	±[b]	+
C. botulinum		
Type A, NCTC7272	+++	++
Type B, NCTC7273	+++	++
Type C, NCTC3732	-[b]	+++
Type E, NCTC8266	+	++
C. difficile		
NCTC11223	++	++
N3	++	++
N6	++	++
MPRL2	++	++
MPRL105	+++	++
MPRL174	++	++

[a] See Materials and Methods for assessment of growth. PPW5 = proteose peptone water; THB = Todd-Hewitt broth.

[b] 96-h culture, 1-ml inoculum; other cultures incubated 48 h.

TABLE IV/VII

Growth of other neuraminidase-negative clostridia obtained in two broth media

Test organism and strain number	Growth in stated medium[a]	
	PPW5	THB
C. paraperfringens		
2227	+	+
3-3	+	+
G	+	++
9	+	+
C. sporogenes		
23	+++	+++
24	+++	+++
26	+++	+++
28	+++	+++
C. histolyticum		
NCTC503	+++	++
NCTC7123	+++	+++
NCTC7124	+++	+++
CH2	+++	+++
C. butyricum		
NCTC7423	±[b]	++
C. sphenoides		
NCTC507	+	+++
C. fallax		
NCTC8380	+++	++
C. tetanomorphum (*C. cochlearium*)		
NCTC2909	++	+++
C. subterminale		
CS1	++	+++

[a] See footnote to table IV/VI.

[b] 96-h culture, 1-ml inoculum; other cultures incubated 48 h.

V. IN-VITRO PRODUCTION OF NEURAMINIDASE BY BACTEROIDACEAE

Va. Production of neuraminidase by Bacteroides fragilis strain NCTC9344

The methods developed for investigation of neuraminidase production by Clostridium perfringens were adapted for studies with Bacteroides fragilis (B. fragilis ss. fragilis) strain NCTC9344. Table V/I gives the results when culture supernates and cell extracts from 48-h cultures in a range of broth media were tested by the standard neuraminidase assay procedure. The culture supernates contained small amounts of enzyme that could be detected when the incubation time of the assays was prolonged to 24 h. Cell extracts prepared from the same cultures contained larger amounts of neuraminidase that gave high values (E_{549}) in 60-min assays. Neither the culture supernates nor the cell extracts had significant NAN-lyase activity that might interfere with the assay for neuraminidase. Enzyme control values (E_{549}) were satisfactorily low with the Bacteroides cell extracts and there were no problems in demonstrating that the assay product had a typical absorption curve for NANA with a peak at 549 nm. Cell extracts were examined as a routine in further studies with Bacteroides species.

The organism grew adequately in all of the media tested. Growth was least good in thioglycollate broth (TGB) and there were difficulties in centrifuging this medium because it contains a low concentration of agar. DB, PPW5 and BM broth media were chosen for

TABLE V/I

Production of neuraminidase by Bacteroides fragilis NCTC9344 grown in various broth media

Culture medium[a]	Degree of growth[b]	Result of neuraminidase assay[c] in stated culture product (E_{549})	
		Culture supernate	Cell Extract
DB	+++	0.112	0.457
PPW5	++	0.076	0.547
BM	++	0.164	0.713
CMB	+++	0.231	0.936
PPY	++	0.175	0.711
TGB	+	0.893	0.737
THB	+++	0.053	0.546

[a] DB = digest broth; PPW5 = proteose peptone water; BM = BM medium; CMB = cooked-meat broth; PPY = PPY medium; TGB = thioglycollate broth; THB = Todd-Hewitt broth.

[b] See Materials and Methods for assessment of growth.

[c] Supernates of 48-h cultures tested in 24-h assays with substrate FVII(8); cell extracts from the same cultures tested in 60-min assays. The spectrophotometric value (E_{549}) is an average result obtained from duplicate assay readings corrected by subtraction of the sum of the values for separate substrate and enzyme control tests.

further studies as media likely to support growth and give good neuraminidase production with a range of Bacteroides species.

A more concentrated bulk cell extract (P15) was prepared from a 200-ml PPW5 culture of B. fragilis NCTC9344 (see Materials and Methods) for studies of the effect of pH and Ca^{2+} on the enzyme. Fig. V/1 shows the results of 30-min neuraminidase assays in acetate buffers at different pH values. The assay values were corrected by subtraction of the values obtained in separate substrate and enzyme control tests at each pH value; the enzyme controls were constant (E_{549} c. 0.02) but the substrate controls were slightly higher at lower pH values (range 0.06-0.11). Fig. V/1 shows the pH optimum for B. fragilis neuraminidase with substrate FVII to be at c. 4.2. Our standard assay uses acetate buffer at pH 5.1; this allows good neuraminidase activity and was therefore used when testing for neuraminidase production by other Bacteroidaceae. The pH values shown in fig. V/1 are the values for the initial pH of the buffers prepared for use in the tests. Direct measurement of pH in equivalent test mixtures showed that the actual pH values might be 0.2-0.3 units higher, but they did not vary during incubation of the tests; thus the pH optimum for B. fragilis neuraminidase in sodium acetate buffers is likely to be c. 4.5.

The influence of Ca^{2+} on the activity of the B. fragilis neuraminidase preparation P15 was tested by the procedures used for the enzymes of C. perfringens and Vibrio cholerae (see table II/IV). Table V/II shows that the addition of Ca^{2+} did not stimulate B. fragilis enzyme activity; the addition of 1mM EDTA did not inhibit the enzyme and even 5mM EDTA reduced the assay value by

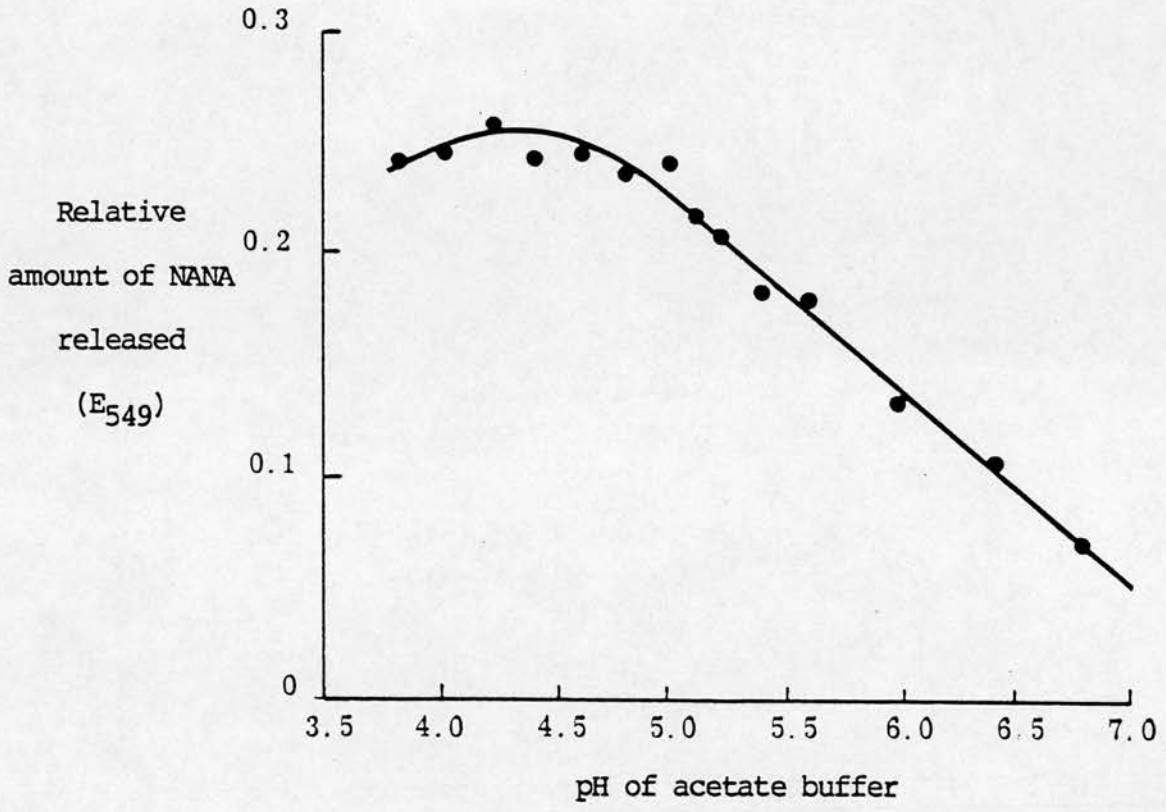


Fig. V/1 - Effect of pH on the assay for the neuraminidase of Bacteroides fragilis. Cell extract of B. fragilis NCTC9344 (P15) incubated with substrate FVII(8) in sodium acetate buffers (see Materials and Methods).

TABLE V/II

Effect of Ca^{2+} and EDTA on the assay of neuraminidase from Bacteroides fragilis NCTC9344

Concentration of added Ca^{2+} or EDTA in reaction mixture[a]	Result of neuraminidase assay[b] (E_{549})
3mM Ca^{2+}	0.186
1mM Ca^{2+}	0.171
None added	0.188
1mM EDTA	0.171
5mM EDTA	0.118

[a] See Materials and Methods.

[b] B. fragilis NCTC9344 cell extract (P15) diluted 1 in 40 in acetate buffer, pH 5.1, was used as test enzyme in standard assay mixtures with substrate FVII(8). The spectrophotometric value (E_{549}) is an average result obtained from duplicate 15-min assay readings corrected by subtraction of the values for the corresponding substrate and enzyme control tests.

c. one third only. Ca^{2+} was not added as a routine to assays for neuraminidase production by species of Bacteroidaceae.

Vb. Production of neuraminidase by species of the B. fragilis group

Table V/III gives the results when a range of strains belonging to the group of species known collectively as the B. fragilis group were tested for neuraminidase production by procedures similar to those developed for testing clostridial species. The purity and identity of each organism was carefully checked as described in Materials and Methods. The strains were cultured at 37°C for 48 h in DB and PPW5 media and the amount of growth was noted. Cell extracts were prepared and tested in standard neuraminidase assays incubated at 37°C for 60 min; samples giving low-positive or negative results were retested in 24-h assays. The detailed procedures and the system used for grading the amount of neuraminidase present are described in Materials and Methods.

In general, these organisms grew better in DB than in PPW5 broth, but neuraminidase-positive strains usually produced greater activity in PPW5. All the strains of B. fragilis (B. fragilis ss. fragilis) and B. vulgatus produced large amounts of the enzyme in both media, and the strains of B. distasonis and B. ovatus were also clearly positive.

All strains of B. thetaiotaomicron produced neuraminidase well in PPW5 but production in DB medium sometimes varied. Strain NCTC10582 was cultured in DB medium on four occasions;

TABLE V/III

Production of neuraminidase by organisms of the Bacteroides fragilis group grown in two broth media

Test organism and strain number	Growth in stated medium[a]		Relative amount of neuraminidase activity in culture product[b]	
	DB	PPW5	DB	PPW5
B. fragilis				
NCTC9343	+++	+++	+	+++
NCTC9344	+++	+++	+++	+++
WPH1	+++	+++	+++	+++
WPH12	+++	+++	+++	+++
WPH21	+++	+++	+++	+++
B. vulgatus				
NCTC10583	+++	+++	+++	+++
GNAB9	+++	+++	++	+++
GNAB29	+++	+++	++	+++
B. distasonis				
ATCC8503	+	++	+	++
GNAB26	++	+	++	+++
GNAB39	++	+	++	+++
B. ovatus				
ATCC8483	+++	+++	±	++
WPH207	+++	+++	±	++
B. thetaiotaomicron				
NCTC10582	+++	++	V	+++[c]
GNAB1	+++	++	++	+++
GNAB2	+++	++	++	+++
GNAB7	+++	++	±	+++
GNAB11	+++	++	++	+++
GNAB16	++	+	V	++[c]
GNAB20	+++	++	++	+++
B. variabilis				
VPI11368	+++	++	-[c]	V
GNAB18	+++	++	+[c]	+++[c]
B. uniformis				
ATCC8492	+++	+++	-	-
VPI11227	+++	++	-	-
GNAB42	+++	+++	-	-

B. eggerthi/... /see next page

TABLE V/III/contd.

Test organism and strain number	Growth in stated medium[a]		Relative amount of neuraminidase activity in culture product[b]	
	DB	PPW5	DB	PPW5
B. eggerthi				
NCTC11155	++	+	-	-
GNAB21	+++	+	-	-
GNAB23	+++	++	-	-
GNAB24	++	+	-	-
GNAB41	++	+	-	-
GNAB43	+++	++	-	-
B. splanchnicus				
NCTC10825	+++	+++	-	-
NCTC10826	+++	+++	-	-

[a] Cultures incubated for 48 h at 37°C; see Materials and Methods for assessment of growth. DB = digest broth; PPW5 = proteose peptone water.

[b] Cell extract from culture in stated medium was tested. See Materials and Methods for grading of neuraminidase activity; V = variable results in different experiments (see text).

[c] Result confirmed in repeat culture.

neuraminidase assays were twice positive (+ and ++) and twice negative even with assays incubated for 24 h. Variable results were also obtained with strain GNAB16, which was once positive (+) but twice negative. By contrast, three separate cultures of each organism in PPW5 broth were consistently positive.

Results with B. variabilis also varied. Strain GNAB18 consistently produced neuraminidase. Strain VPI11368 twice produced moderate amounts (++) in PPW5 medium but failed, however, to produce detectable activity in one other PPW5 culture and in three cultures in DB medium.

All strains of B. uniformis, B. eggerthi and B. splanchnicus grew adequately but none produced detectable neuraminidase activity in either medium, even when tested in 24-h assays. As a routine in all these studies it was confirmed that negative results were not attributable to NAN-lyase activity in the cell extract preparations. Similarly, the position of the absorption peak at 549 nm was confirmed for all positive neuraminidase assays.

Vc. Production of neuraminidase by other Bacteroides species

The results of similar tests with other Bacteroides species are shown in table V/IV; the current classification of these strains is described in Materials and Methods. Most strains grew adequately in DB and BM media in 48 h; a few cultures required 96-h incubation for adequate growth. Since many of the strains grew very poorly or not at all in PPW5 broth even when incubated

TABLE V/IV

Production of neuraminidase by strains of various Bacteroides species grown in broth media

Test organism and strain number	Growth in stated medium[a]			Relative amount of neuraminidase activity in culture product[b]	
	DB	BM	PPW5	DB	BM
B. melaninogenicus ss. melaninogenicus					
ATCC15930	++	++	+[c]	++	+++
VPI4196	++	+++	++	±	++
WPH67	+++	+++	-[c]	V	-[d]
B. melaninogenicus ss. levi					
VPI3300	+++	+++	-[c]	+++	+++
B. melaninogenicus ss. intermedius					
NCTC9336	+++	++	-[c]	-	-
NCTC9338	++	+[c]	-[c]	-	-
WPH4	+++	+++	-[c]	-	-
WPH26	+++	++[c]	-[c]	-	-
WPH31	++	++	-[c]	-	-
B. oralis					
VPI9958	++	...	-[c]	+++	...
GNAB46	++	...	+[c]	+	...
GNAB54	+	...	+	+	...
B. buccalis					
VPI8906D	++	++	-[c]	++	++
B. pentosaceus					
NP333	++	++	-[c]	-	-
B. bivius					
VPI5540	+	++	±[c]	+	++
VPI6318	+	...	+[c]	+	...
VPI6822	++	...	+[c]	++	...
VPI7880	++	...	+	++	...
B. ruminicola					
B38024	+++	+	-[c]	-	-
B38080	+	+	-[c]	-	-
B56029	+	+	-[c]	-	-

B. disiens/... /see next page

TABLE V/IV/contd.

Test organism and strain number	Growth in stated medium[a]			Relative amount of neuraminidase activity in culture product[b]	
	DB	BM	PPW5	DB	BM
B. disiens					
VPI7852	+	+++	+	-	-
VPI8057	+++	+++	+++	-	-
B. asaccharolyticus					
NCTC9337	++[c]	++	-[c]	-	-
WPH30	++	++	-[c]	-	-
WPH57	++	+	-[c]	-	-
B. gingivalis					
WPH15	+[c]	++	-[c]	-	-
B. corrodens					
143A	+[c]	±[c]	+	-	-
151RV	+[c]	±[c]	+[c]	-	-

[a] See Materials and Methods for assessment of growth; ... = not tested. DB = digest broth; BM = BM broth; PPW5 = proteose peptone water.

[b] See footnote to table V/III; ... = not tested.

[c] Culture incubated 96 h; other cultures incubated 48 h.

[d] Result confirmed in repeat culture.

for 96 h, the cultures in this medium were not tested for neuraminidase as a routine.

The three strains of B. melaninogenicus ss. melaninogenicus and the single strain of B. melaninogenicus ss. levi produced neuraminidase. All were consistently positive except strain WPH67 which proved variable; it twice gave ++ production in DB medium, but was negative on one other occasion and was twice negative in BM medium. By contrast, the five strains of B. melaninogenicus ss. intermedius were negative in both media.

The strains of B. oralis and B. bivius were all neuraminidase-positive as was strain VPI5906D, now allocated to the new species B. buccalis. The strains of B. pentosaceus, B. ruminicola, B. disiens, B. asaccharolyticus, B. gingivalis and B. corrodens (B. ureolyticus) were all neuraminidase-negative. The B. corrodens strains did not grow well in BM broth; they were also tested and found to be negative in PPW5 medium.

Vd. Studies with Capnocytophaga, Fusobacterium and Leptotrichia species

Strains of other genera of Gram-negative anaerobic non-sporing bacilli were examined for neuraminidase production by the same procedures as were used for Bacteroides species. Table V/V shows that all the strains of Capnocytophaga (previously classified as B. ochraceus; see Materials and Methods) could produce neuraminidase. The strains grew best in DB medium and this medium gave the best production of neuraminidase. Four strains produced the enzyme well in DB medium; however, their enzyme production in

TABLE V/V

Production of neuraminidase by strains of

Capnocytophaga ochracea grown in three broth media

Strain number	Growth in stated medium[a]			Relative amount of neuraminidase activity[b] in culture product		
	DB	BM	PPW5	DB	BM	PPW5
10	++	+	-[c]	++	±	...
73	++	++	+	++	±	±
1956C	+++	+	+	+++[d]	-	-[d]
2467B	+++	++	++[c]	++	-	-
VPI2845	++	+	+[c]	V	-[d]	-
79B	+++	+	±[c]	±[d]	V	-

[a] See Materials and Methods for assessment of growth. DB = digest broth; BM = BM medium; PPW5 = proteose peptone water.

[b] Cell extract from culture in stated medium was tested. See Materials and Methods for grading of neuraminidase activity; ... = not tested. V = variable results in different experiments (see text).

[c] Culture incubated 96 h; other cultures incubated 48 h.

[d] Result confirmed in repeat culture.

BM and PPW5 media was very unreliable. Strain 79B produced trace amounts ($\pm$ score) on three occasions in DB medium and on one further occasion in BM broth. Strain VPI2845 produced a trace of activity in only one of three cultures in DB medium and was consistently negative in BM and PPW5 cultures. The difficulty in demonstrating neuraminidase activity in these cultures may be partly explained by the presence of small amounts of NANA-destroying activity in the cell extract of several of the Capnocytophaga cultures.

Table V/VI shows the results when eight strains of Fusobacterium species and one of Leptotrichia buccalis were grown in DB and PPW5 medium; in general, they grew well in DB and less well in PPW5. The two strains that grew poorly in PPW5 were also grown and tested in BM medium. Cell extracts of all cultures were neuraminidase-negative.

TABLE V/VI

Growth of neuraminidase-negative Fusobacterium and Leptotrichia species obtained in three broth media

Test organism and strain number	Growth in stated medium[a]		
	DB	PPW5	BM
F. necrophorum			
NCTC10575	+++	++	...
NCTC10576	++	+	...
NCTC10577	++	+	...
F. nucleatum (*F. polymorphum*)			
NCTC10562	+++	+	...
WPH142	++	+	...
WPH160	++	±[b]	+++
F. necrogenes			
NCTC10723	+[b]	+[b]	...
F. varium			
NCTC10560	++	+	...
L. buccalis			
NCTC10249	++	-[b]	+

[a] See Materials and Methods for assessment of growth; ... = not tested. DB = digest broth; PPW5 = proteose peptone water; BM = BM broth.

[b] Culture incubated 96 h; other cultures incubated 48 h.

VI. STUDIES WITH ANTISERA

VIa. Assay for anti-neuraminidase activity in serum

The standard neuraminidase assay was adapted to allow measurement of the degree of enzyme inhibition by various antisera (see Materials and Methods). Test sera were allowed to react with a standard C. perfringens type A neuraminidase preparation for 30 min at room temperature in order to allow neutralisation of the enzyme before the start of the neuraminidase assay. Table VI/I gives the results of an experiment to determine the degree of inhibition of neuraminidase preparation P9D4 by C. perfringens diagnostic antiserum (CPA1), a commercially available equine antiserum prepared by injecting C. perfringens type A culture products in order to produce high-titre anti-phospholipase-C activity (see Materials and Methods). Assays were incubated for 15 and 30 min; time-zero (T_0) assays were also performed. The assay values for P9D4 in the absence of antiserum show the expected doubling in value when incubation was prolonged from 15 to 30 min. The C. perfringens CPA1 serum produced a marked reduction in assay value after each of these periods of incubation, the degree of inhibition remaining approximately constant at c. 85%. It is apparent that this antiserum, although prepared primarily for its anti-phospholipase activity, also has considerable anti-neuraminidase activity.

The T_0 enzyme control value was higher for the reaction mixture that contained antiserum than for the reference assay with

TABLE VI/I

Inhibition of Clostridium perfringens type A neuraminidase by C. perfringens CPA antiserum

Test mixture[a]	Time of incubation at 37°C before start of assay for NANA (min)	Spectrophotometric reading[b] (E_{549})			Inhibition of neuraminidase activity[d] (%)
		Test	Enzyme control	Corrected value[c]	
P9D4 + saline	0	0.025	0.003	0.002	...
	15	0.218	0.006	0.192	...
	30	0.427	0.007	0.400	...
P9D4 + CPA1	0	0.047	0.028	0.000	...
	15	0.101	0.048	0.033	83
	30	0.144	0.066	0.058	86

[a] Enzyme preparation P9D4 allowed to react with undiluted CPA1 serum or with saline for 30 min at room temperature before the assay for neuraminidase. For assay mixtures, see Materials and Methods.

[b] Average of duplicate assay readings.

[c] Test readings corrected by subtraction of the sum of the values for the FVII(7) substrate control (E_{549} = 0.020) and the appropriate enzyme control assays.

[d] Inhibition of enzyme activity by serum expressed as a percentage of the value for the equivalent test with no added antiserum; ... = not applicable.

the serum replaced by saline, and these enzyme control values increased progressively during incubation of the tests; it was confirmed that the 30-min enzyme control value (E_{549} = 0.066) was due to the production of a chromogen with the typical absorption spectrum of NANA (peak absorption 549 nm). Thus, test sera may contain glycoprotein that can itself act as substrate for the enzyme during these assays. It is possible that this experiment underestimates the extent of this, since the good anti-neuraminidase activity of serum CPA1 might be expected to inhibit the enzyme's action; however, similar assays with other equine antisera that contain very low inhibitory activity for C. perfringens neuraminidase (e.g. C. tetani ATS serum, see below) gave only marginally higher 30-min enzyme control values. It is important to correct test results for undiluted sera by subtraction of the values for separate 30-min enzyme and substrate control assays; the use of the T_0 control assay for correcting the test values in table VI/I would give misleadingly high assay values (and hence an underestimate of the inhibitory action of the antiserum). This effect is not seen in our standard anti-neuraminidase assays with sera diluted 1 in 100, when enzyme control levels are very low and do not rise during incubation at 37°C for 30 min.

Fig. VI/1 shows the effect of two equine antisera on the release of NANA from the FVII substrate by the enzyme preparation P9D4 in assays incubated for periods up to 1 h (see Materials and Methods for details of sera). The ATS serum, an experimental antiserum raised against C. tetani toxin, was included as a control serum that should not contain specific antibody against C. perfringens neuraminidase. Serum 1863 was an experimental antiserum

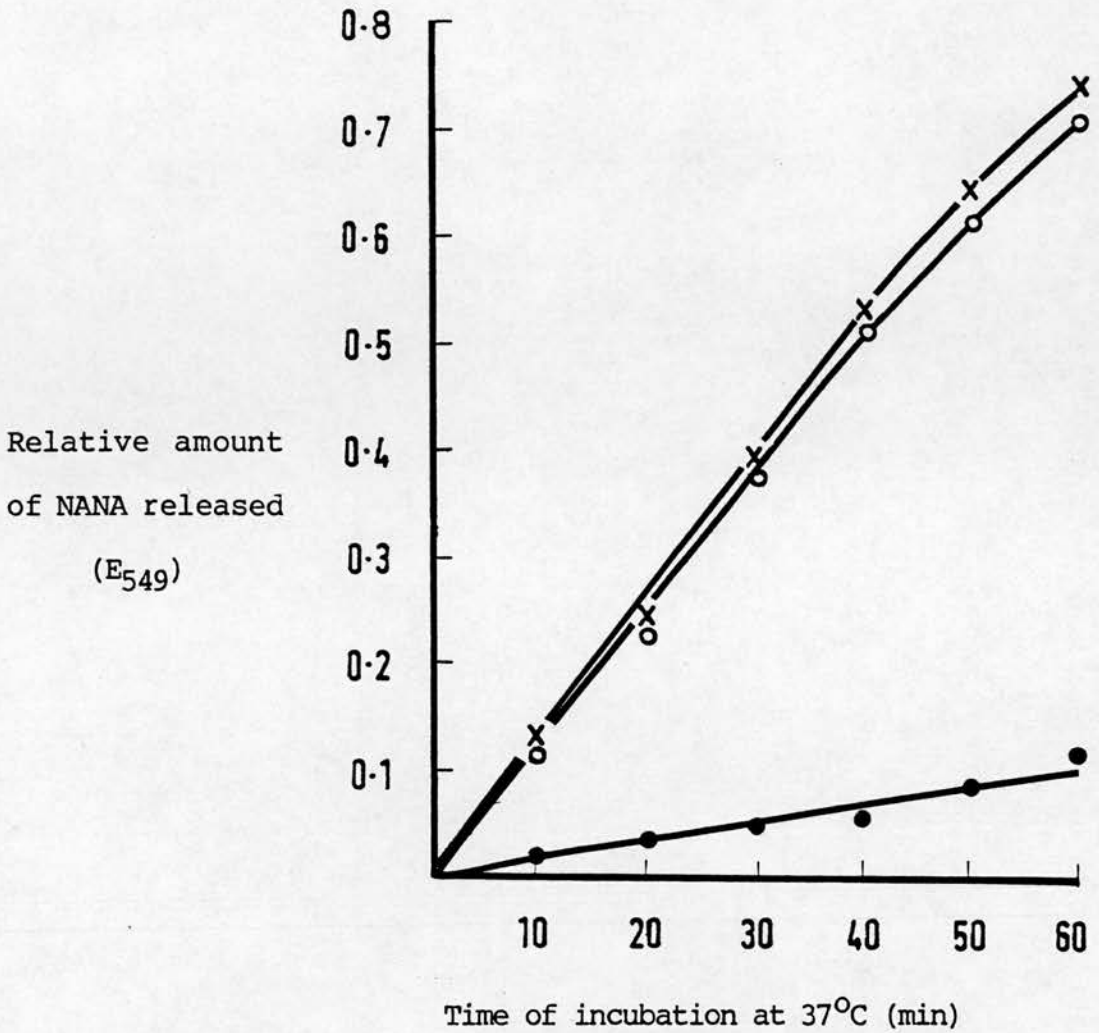


Fig. VI/I - Release of NANA from substrate FVII(7) by Clostridium perfringens neuraminidase preparation P9D4 after neutralisation by two antisera. Sera, diluted 1 in 100 in saline, allowed to react with enzyme for 30 min at room temperature before the assay for neuraminidase. Test results corrected by subtraction of the value for the appropriate T_0 assay. Test sample: ●—●, P9D4 + C. perfringens 1863 serum; o—o, P9D4 + C. tetani ATS serum; x—x, P9D4 + saline.

raised against C. perfringens type A culture products that was found to have a good level of anti-neuraminidase activity. Both antisera were tested at a dilution of 1 in 100 in saline. The reference assays of enzyme activity with no added antiserum show the expected linear release of NANA up to values (E_{549}) of c. 0.5. The C. tetani ATS serum had very little effect on the activity of the enzyme but the C. perfringens 1863 serum gave marked inhibition (c. 85%) and this remained constant regardless of the period of incubation of the assays. The incubation time for further tests of neuraminidase inhibition was standardised at 30 min; this gave reliable reference enzyme assay values (E_{549}) of c. 0.4 with enzyme preparations P9D3 or P9D4.

Fig. VI/2 shows the titration curves obtained when serial dilutions of these equine sera were assayed for anti-neuraminidase activity in standard 30-min tests. Undiluted C. perfringens 1863 serum gave complete inhibition of the neuraminidase preparation but there was a steep fall in the amount of inhibitory activity when dilutions greater than 1 in 4 were tested; on this occasion the value for a dilution of 1 in 100 (c. 55%) would appear to be somewhat lower than was found in the previous experiment. The C. perfringens CPA1 serum was also active against the test enzyme but it did not give complete inhibition even when tested undilute; the value here (84%) is similar to that obtained in the experiment detailed in table VI/I. It produced rather less inhibition than serum 1863 over most of the range of dilutions tested, though the slope of the curve appears a little less steep. By contrast, the undiluted C. tetani ATS serum gave only 22% inhibition and this

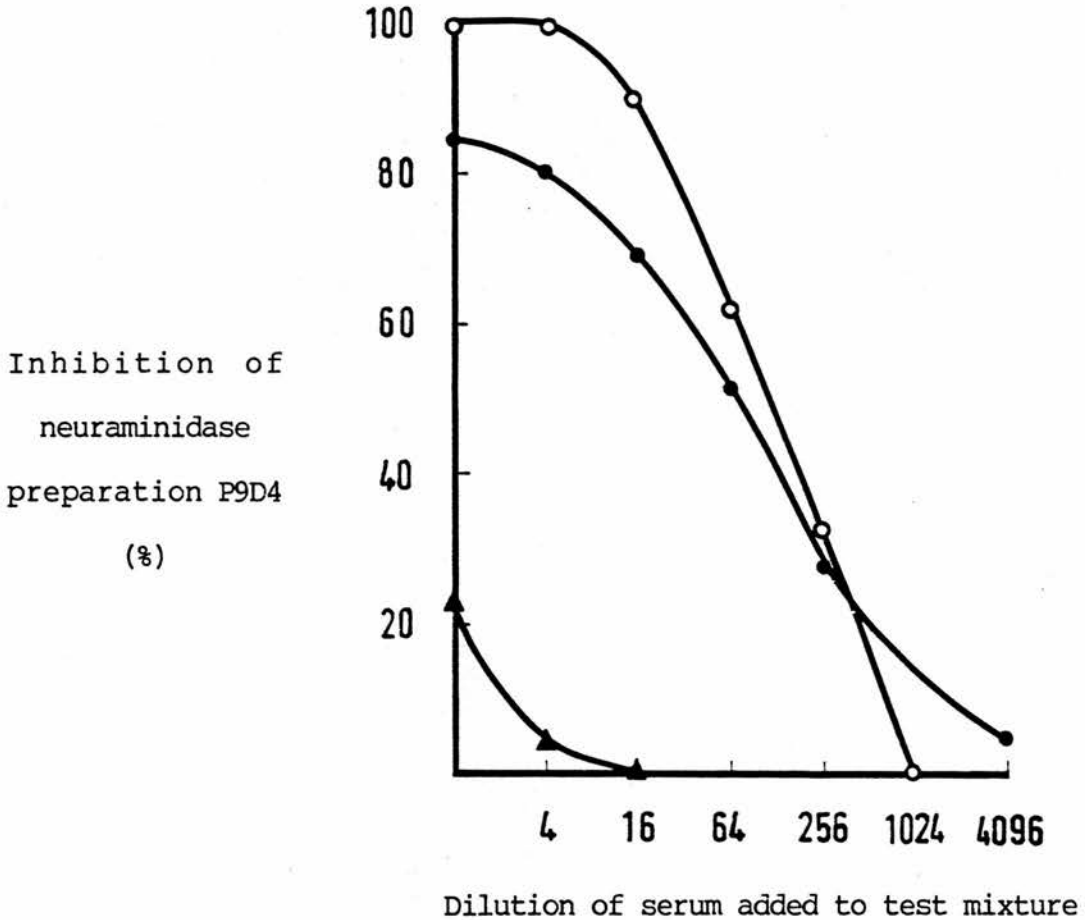


Fig. VI/2 - Inhibition of Clostridium perfringens neuraminidase by serial dilutions of three equine antisera. Neutralisation period 30 min at room temperature. Test sera; ●—●, C. perfringens CPA1; o—o, C. perfringens 1863; ▲—▲, C. tetani ATS (see Materials and Methods).

fell rapidly to insignificant levels on dilution. It seems probable that undiluted horse serum contains glycoproteins that may act as non-specific inhibitors of neuraminidase; all sera should be diluted at least 1 in 10 before assay to avoid this problem.

It is conventional to express the antibody content of serum as a titre - the reciprocal of the greatest dilution that will produce a particular observable effect, e.g. 100% or 50% inhibition of the observed activity. It is apparent from fig. VI/2, and from similar curves prepared for other serum samples (e.g. see figs VI/8, 10 and 13), that it would be difficult to express the inhibitory effect of these sera as a simple titre even when these full titration curves are prepared. It is not uncommon for the slopes of such curves to vary so that the lines intersect (e.g. see figs VI/10 and 13); in such cases the relative efficiencies of the two sera are reversed at high and low dilutions. A further problem is that the preparation of such titration curves requires so many neuraminidase assays that only two or three sera can be assessed in a single batch of tests and this makes it very difficult to include control tests to confirm the comparability of results obtained on different occasions. In practice it proved much simpler to compare sera by assessing the degree of inhibition produced at the standard dilution of 1 in 100; thereafter only a limited number of sera were examined in greater detail in assays performed with serial serum dilutions.

Fig. VI/3 gives the results of an experiment performed to determine whether our standard assay procedure allows adequate neutralisation of the enzyme by antiserum. Serial dilutions of the C. perfringens 1863 antiserum were allowed to react with enzyme

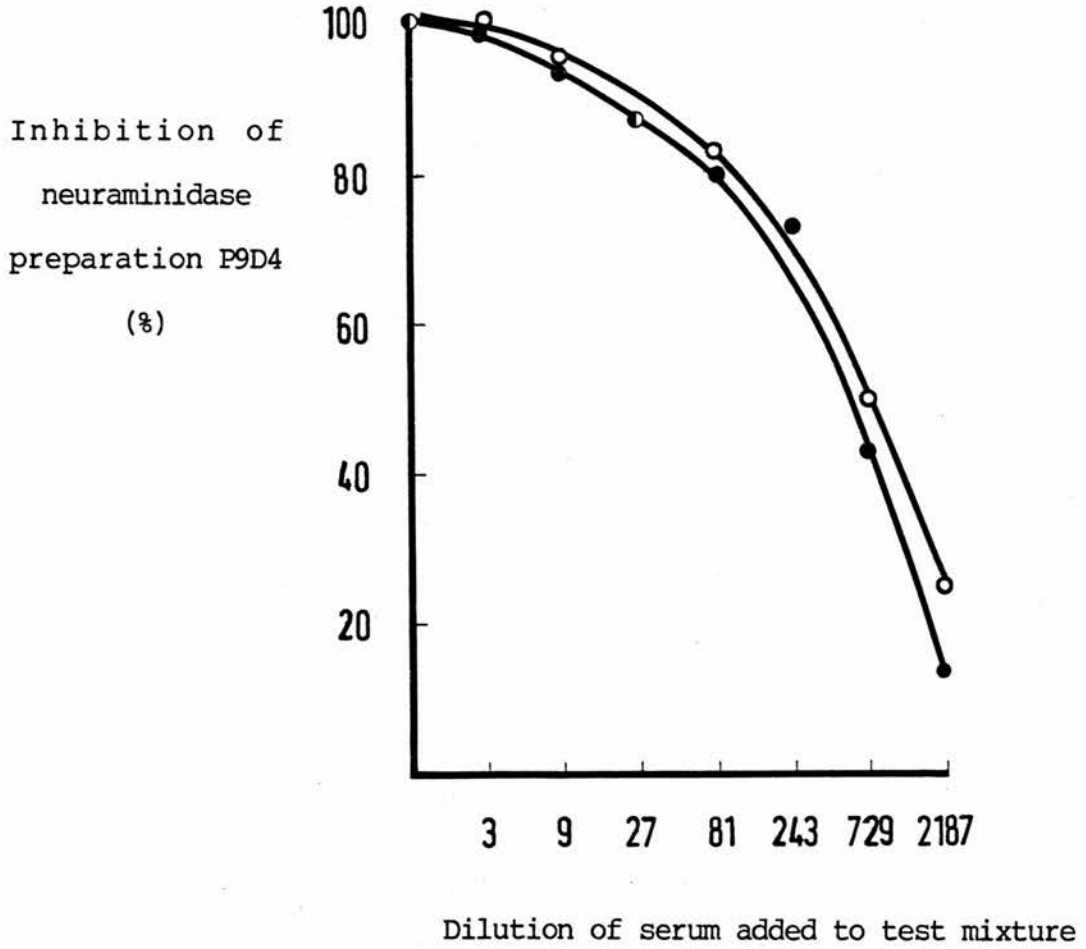


Fig. VI/3 - Comparison of two procedures for neutralisation of Clostridium perfringens neuraminidase by serial dilutions of C. perfringens 1863 antiserum. Conditions for neutralisation: ●—●, 30 min at room temperature; o—o, 20 h at 4°C.

preparation P9D4 either for 20 h at 4°C, or for 30 min at room temperature, before standard assays for neuraminidase activity with FVII(7) substrate. It is apparent that there is very little difference between the results obtained with the two procedures; our standard neutralisation procedure (30 min at room temperature) was adopted because of its greater convenience.

On this occasion the value for the inhibitory activity of serum 1863 at a dilution of 1 in 100 was c. 80%; this agrees well with the value obtained in fig. VI/1. In general, assays for anti-neuraminidase activity performed on different occasions with either P9D3 or P9D4 as standard enzyme preparation proved reasonably consistent (e.g. see similar titration curves for serum 1863 in figs VI/8, 10 and 13). However, occasional batches of assays gave results that were generally higher or lower; for this reason it is advisable to include a standard serum of known inhibitory activity with each batch of assays.

VIb. Production of concentrated neuraminidase preparations from PPW5 culture supernate of C. perfringens strain L2Ab for rabbit immunisation

In order to produce concentrated preparations of neuraminidase from C. perfringens strain L2Ab, a further bulk PPW5 culture was prepared by the same procedure as was used for the stock P9 neuraminidase preparation. The supernate from this culture (P12) was fractionated with ammonium sulphate in two batches as described in Materials and Methods. Table VI/II summarises the results of assays for protein and neuraminidase in

TABLE VI/II

Neuraminidase content of samples prepared from the bulk PPW5 culture supernate (P12) of Clostridium perfringens strain L2Ab by ammonium sulphate fractionation

Sample[a]	Volume (ml)	Protein[b]		Neuraminidase		
		Concentration (mg/ml)	Total (g)	Concentration[c] (milli-units/ml)	Total (units)	Specific activity (units/g protein)
A	500	25.8	...	40	20.0	...
A_{50}	88	4.8	0.4	34	3.0	7.5
A_{85}	9.0	148.2	1.3	1795	16.2	12.5
B	580	21.5	...	42	24.4	...
B(dial)	685	6.4	4.4	30	20.6	4.7
B_{50}	4.7	21.8	0.1	1329	6.2	62.0
B_{85}	8.0	146.0	1.2	1241	9.9	8.3

[a] Precipitates obtained at 50% and 85% $(NH_4)_2SO_4$ saturation with two batches (A and B) of the P12 culture supernate; batch B was centrifuged and dialysed before fractionation (see Materials and Methods).

[b] Protein estimations in undialysed samples A and B were falsely high and not used for further calculations (see text).

[c] Values for neuraminidase activity calculated from the results (E_{549}) of 60-min assays for appropriate dilutions of each sample with FVII(11) substrate by comparison with the value obtained on assay of the reference sample of 10 µg NANA (see footnote to table II/V).

the dialysed fractions prepared from the precipitates obtained at 50% and 85% saturation. The assay used for estimation of the protein content of the undialysed P12 samples also detects smaller peptone molecules present in the PPW5 culture medium; the lower value obtained for the dialysed sample of batch B is likely to give a more accurate estimate of the protein present in the starting material. The neuraminidase activity in the various samples was expressed as the observed rate of release of NANA (μmoles/min) from substrate FVII under the conditions of our standard assay. The sample containing the precipitate from batch A at 50% $(NH_4)_2SO_4$ saturation (A_{50}) contained only c. 15% of the total neuraminidase in the starting material; it was not further concentrated and was discarded because the specific activity of the enzyme in this fraction had not been significantly increased. The bulk of the enzyme (c. 80%) was present in the precipitate obtained on increasing the $(NH_4)_2SO_4$ concentration from 50% to 85% saturation; the concentrated preparation from this fraction had high neuraminidase activity and a specific activity of 12.5 units/g protein.

The pattern of neuraminidase precipitation was slightly different when the dialysed preparation of the second batch of P12 was similarly fractionated. In this case a higher proportion (c. 30%) of the enzyme was present in the 50% precipitate (B_{50}); the concentrated preparation from this fraction contained relatively little protein and the specific activity was much higher (62 units/g). When the $(NH_4)_2SO_4$ concentration in batch B was increased to 85% saturation the precipitate (B_{85}) contained c. 50% of the total enzyme; the concentrated preparation from this

fraction had high enzyme activity but its specific activity was rather low (8.3 units/g). It seems probable that greater purification of neuraminidase might be obtained with fractions produced by increasing the concentrations of $(NH_4)_2SO_4$ in smaller steps around the 50% saturation level. As the present fractionation procedure produced several preparations that contained moderate amounts of partially purified enzyme in relatively high concentration, these were used for rabbit immunisation studies (see below).

VIc. Immunisation of rabbits with C. perfringens neuraminidase

Attempts were made to produce high-titre anti-neuraminidase sera by immunising rabbits with various preparations of neuraminidase. The results for each of five rabbits immunised by different procedures are presented below. In each case samples of blood were taken at intervals and the anti-neuraminidase activity was monitored in standard assays with serum diluted 1 in 100. The values obtained for an individual serum sample might vary a little in different batches of assays on different occasions; a selection of serum samples was assayed on each occasion in order to obtain an accurate picture of the trend of the current response as a guide to decisions about the immunisation procedure. The graphs showing the overall picture of antibody response for each rabbit (see below) are constructed with values taken from batches of tests performed on a number of separate occasions that gave values that were consistent with each other; the results of many other batches of

assays that also included many intermediate serum samples confirmed the accuracy of the general patterns shown in these graphs.

Rabbits 1 and 2. Two rabbits were given injections of the concentrated preparations of C. perfringens strain L2Ab neuraminidase described in Section VIb. Each rabbit was given a series of three injections of antigen mixed with an equal volume of adjuvant; the details of each injection are shown in table VI/III. It should be appreciated that the values given for neuraminidase content of these injections are calculated from the observed activity with FVII substrate under our standard assay conditions but that they may not be directly comparable with those given below for the Sigma enzyme used in later immunisation studies (see Section IIf for comparison of these assay systems). The initial injection to each rabbit contained the A_{85} fraction (see table VI/II) with incomplete Freund's adjuvant and a second injection of the same mixture was given three weeks later. Fig. VI/4 shows that there was very little response to these injections; the anti-neuraminidase activity remained below 20% and much of the apparent fluctuation in level is likely to be due to variability of the assay at these low levels. A third injection, with complete Freund's adjuvant, was given to each animal after 10 weeks. For this injection, Rabbit 1 was given the more purified B_{50} fraction whereas Rabbit 2 was given the rather weaker B_{85} fraction; neither animal showed more than a transient low-level response. Titration curves prepared for the serum samples at week 14 (fig. VI/5) confirmed that they contained very little anti-neuraminidase activity.

TABLE VI/III

Schedule of immunisation of Rabbits 1 and 2 with Clostridium perfringens strain L2Ab neuraminidase preparations

No. of injection[a]	Adjuvant preparation[b]	Neuraminidase preparation[c]	Dose of neuraminidase[d] (units)	Dose of protein[d] (mg)
Rabbit 1				
1	FI	A_{85}	1.6	130
2	FI	A_{85}	1.4	111
3	FC	B_{50}	2.3	39
Rabbit 2				
1	FI	A_{85}	1.5	120
2	FI	A_{85}	1.8	148
3	FC	B_{85}	0.9	110

[a] All injections subcutaneously; for timing of injections see fig. VI/4.

[b] FI and FC = incomplete and complete Freund's adjuvant respectively.

[c] For neuraminidase preparations see table VI/II.

[d] The amounts of neuraminidase and protein given in each injection calculated from the values in table VI/II.

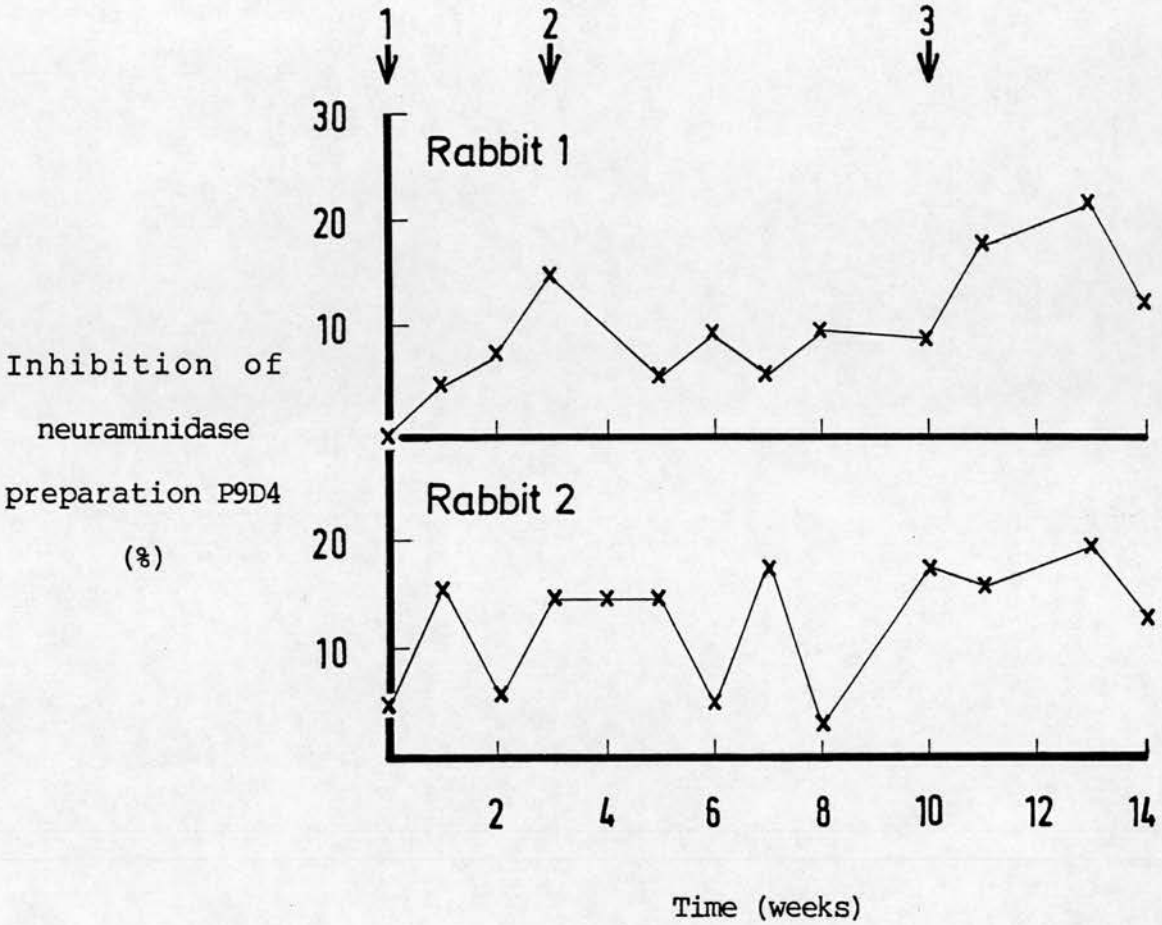


Fig. VI/4 - Immunisation of Rabbits 1 and 2 with Clostridium perfringens strain L2Ab neuraminidase (see text); the arrows indicate antigen injections. Anti-neuraminidase response measured as inhibition of C. perfringens neuraminidase preparation P9D4 by test serum samples diluted 1 in 100 before assay.

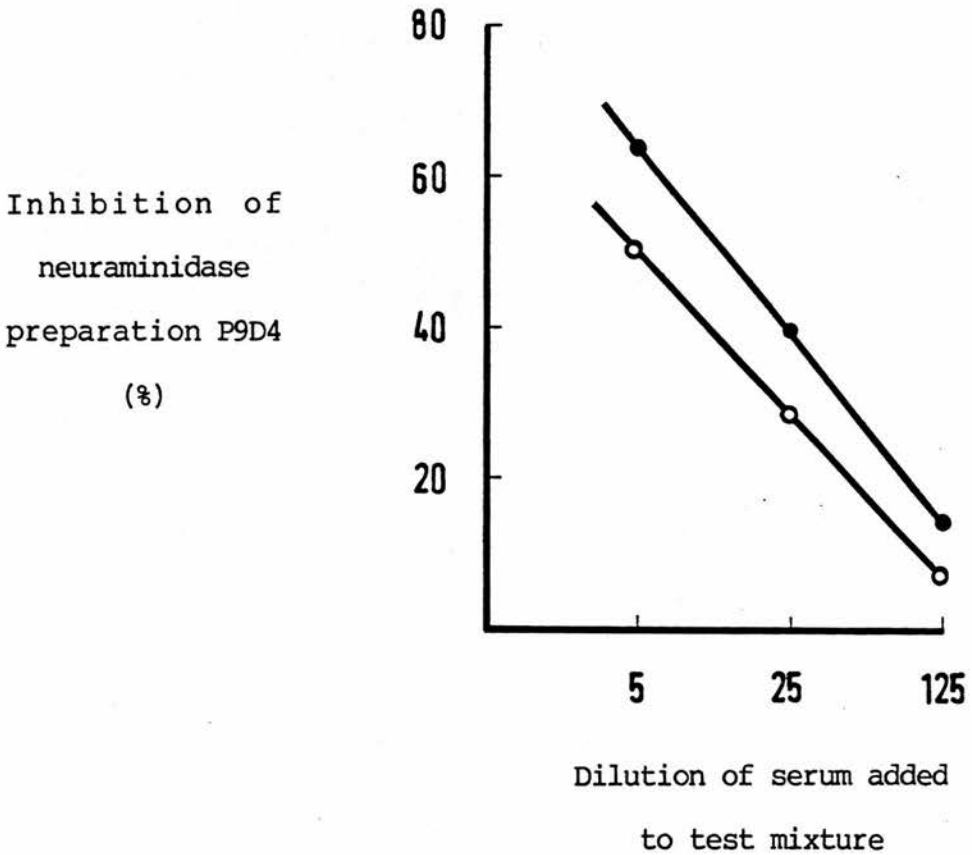


Fig. VI/5 - Inhibition of Clostridium perfringens neuraminidase by serial dilutions of two rabbit antisera: ●—●, Rabbit-1 week-14 serum; o—o, Rabbit-2 week-14 serum.

Fig. VI/6 shows the precipitation lines obtained when the Rabbit-1 week-14 serum was tested against serial dilutions of the A_{85} antigen preparation in a gel-diffusion plate. A major band of precipitation was seen with each of the antigen concentrations tested and at least three other precipitation lines could be distinguished with the antigen diluted 1 in 4, where the best resolution occurred. Similar tests were performed with two other sera known to have much greater anti-neuraminidase activity (serum 1863 and the pooled Rabbit-4 weeks 23-32 antiserum; see Section VIa and fig. VI/10); there was no line of precipitation with either serum. Thus, although the Rabbit-1 serum contained antibodies to at least four components in our crude antigen preparation, it is likely that none of the precipitation lines seen in the gel-diffusion plate was due to antibody with neuraminidase-inhibitory activity. It is probable that our crude neuraminidase preparations contained other proteins that acted as better antigens in these animals. Since the rabbits were already primed to respond to these antigens they would be unlikely to give a good anti-neuraminidase response even if further challenged with purer preparations. The rabbits were killed and the sera discarded; no further attempts were made to produce anti-neuraminidase with these crude enzyme preparations.

Rabbit 3 was immunised with three identical subcutaneous injections containing Freund's incomplete adjuvant and batch 1 of the commercially purified Sigma C. perfringens neuraminidase (see Materials and Methods). The calculated enzyme and protein content of each injection is given in table VI/IV and the course of

Fig VI/6 - Gel-diffusion analysis of serum from Rabbit 1 (see Materials and Methods). Centre well; Rabbit-1 week-14 serum. Peripheral wells; serial dilutions of Clostridium perfringens strain L2Ab neuraminidase preparation A_{85} in distilled water. At least 4 lines of precipitate were formed with the antigen dilution 1 in 4.

TABLE VI/IV

Schedule of immunisation of Rabbits 3, 4 and 5 with Clostridium perfringens Sigma neuraminidase

No. of injection	Route of injection[a]	Adjuvant preparation[b]	Batch of neuraminidase[c]	Dose of neuraminidase (units)	Dose of protein (mg)
Rabbit 3					
1, 2, 3	SC	FI	1	2.1	1.0
Rabbit 4					
1	SC	FC	1	2.1	1.0
2	SC	FC	2	2.1	0.4
3	SC	FC	2	3.2	0.6
4	IV	A	2	5.4	1.0
Rabbit 5					
1	SC	FC	3	5.0	0.8
2	IP	A	3	5.0	0.8
3	IV	A	3	4.2	0.7

[a] SC = subcutaneous; IV = intravenous; IP = intraperitoneal. For timing of injections see figs VI/7, 9 and 11.

[b] See footnote to table VI/III; A = aluminium hydroxide.

[c] See Materials and Methods for enzyme and protein content of the three batches of Sigma neuraminidase.

immunisation is shown in fig. VI/7. The first injection produced a slow rise in antibody to a level of c. 50% inhibition after 6 or 7 weeks but there was no further increase in response to the second and third injections; thereafter there was a slow decline until the inhibitory level was only c. 40% when the animal was killed after 26 weeks. The samples taken between weeks 8 and 11 were pooled in order to give a larger sample representative of the most active serum produced by this animal. The titration curves in fig. VI/8 show that this serum had less inhibitory activity than the experimental C. perfringens 1863 serum over the whole range of concentrations tested; the shape of the early part of the rabbit-serum curve suggests that the activity of the undiluted sample may be partly attributable to the non-specific inhibition noted previously (Section VIa).

Immunisation with the purified Sigma enzyme preparation clearly gave a better response than was obtained with the cruder preparations given to the first two animals although the inhibitory activity produced was still distinctly less than that found in the 1863 serum. The doses of neuraminidase given to this animal (2.1 units/injection) were a little greater than those given to the previous animals but because of the considerably greater purity they contained much less protein (1 mg; see tables VI/III and IV). It seems that the enzyme is a poor antigen since it produced a very slow primary response and no enhancement occurred with further antigen administration. The lack of a secondary response could have been due to the presence of circulating antibody at the time of the repeat injections; this might prevent the effective presentation of the antigen to the animal's immune system.

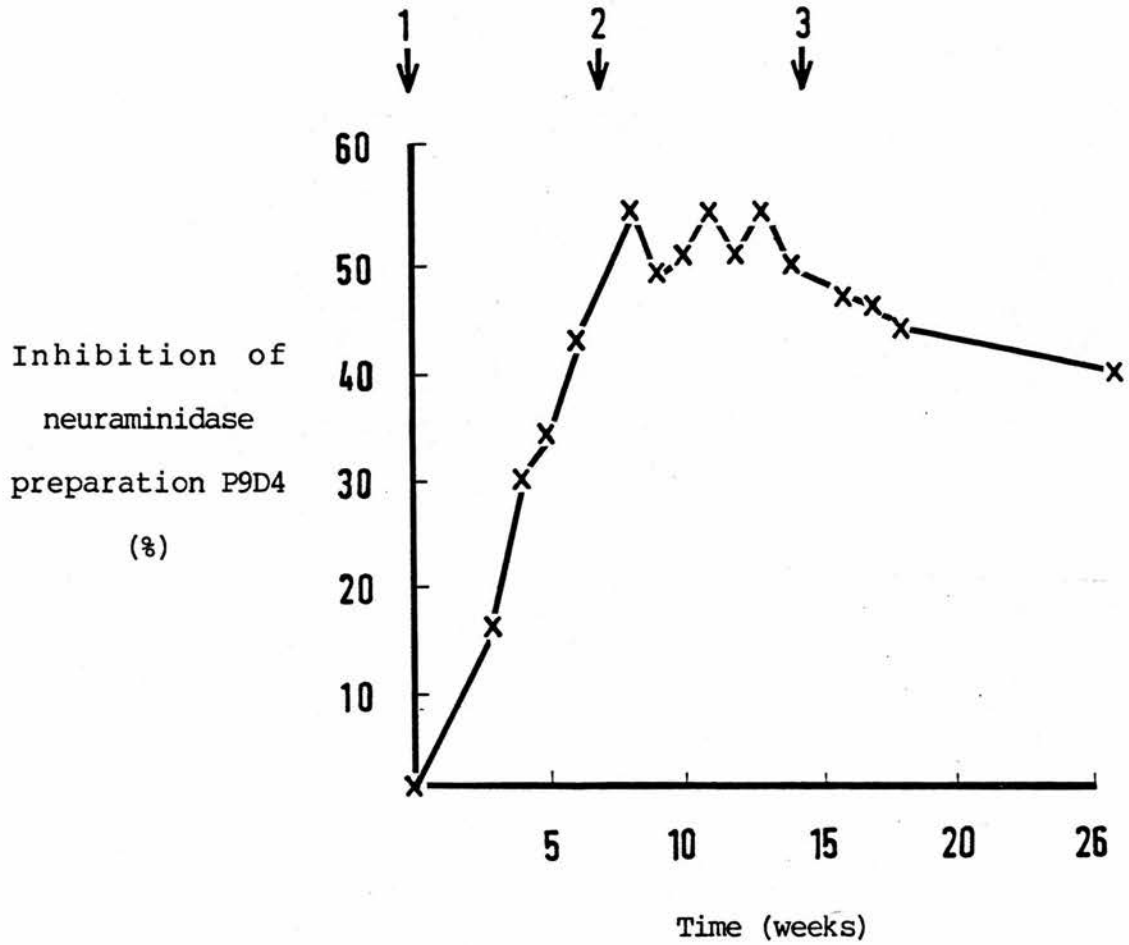


Fig. VI/7 - Immunisation of Rabbit 3 with Clostridium perfringens Sigma neuraminidase (see text); the arrows indicate antigen injections. Anti-neuraminidase response measured as inhibition of C. perfringens neuraminidase preparation P9D4 by test serum samples diluted 1 in 100 before assay.

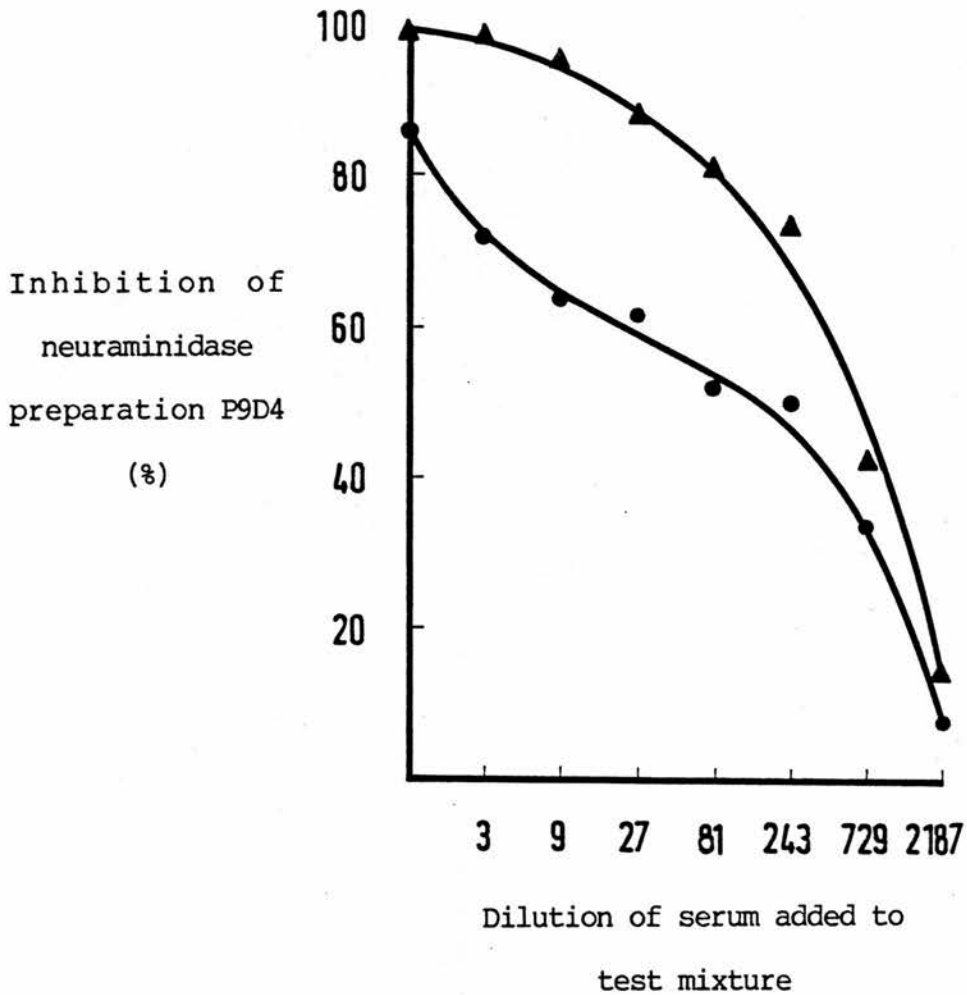


Fig. VI/8 - Inhibition of Clostridium perfringens neuraminidase by serial dilutions of two antisera: ●—●, Rabbit-3 weeks 8-11 pooled serum; ▲—▲, C. perfringens 1863 serum (see text).

Rabbit 4 was also immunised by giving subcutaneous injections of the purified Sigma neuraminidase preparation; details are given in table VI/IV and fig. VI/9. The first injection was similar to that given to Rabbit 3 but with the complete Freund's adjuvant; however, the primary response was less good than on the previous occasion. There was no secondary response to a further injection which contained the same amount (2.1 units) of neuraminidase (but less protein because of the greater specific activity of this batch of enzyme). The antibody level was allowed to decline slowly until the inhibitory level was <10% before the third injection (3.2 units enzyme) was given. The inhibitory level rose steadily again over the next 6 weeks but reached a plateau at c. 60%. A final attempt was made to boost the antibody response by giving a large dose of enzyme (5.4 units) intravenously with aluminium hydroxide as adjuvant. Unfortunately the animal became unwell and died 36 h later, perhaps because of a hypersensitivity reaction provoked by the circulating antibody.

No final serum was obtained but the samples taken between weeks 23 and 32 were pooled. The titration curves in fig. VI/10 confirm that the pooled Rabbit-4 serum had less anti-neuraminidase activity than the C. perfringens equine serum 1863 over most of the range of concentrations tested, though it appears that there was little difference in activity between the two sera in high dilutions. The curve for the pre-inoculation serum again suggests non-specific inhibition with the undiluted normal serum. This further attempt to immunise with higher doses of Sigma neuraminidase and complete Freund's adjuvant was only a little more successful than the previous attempt, although it did suggest that

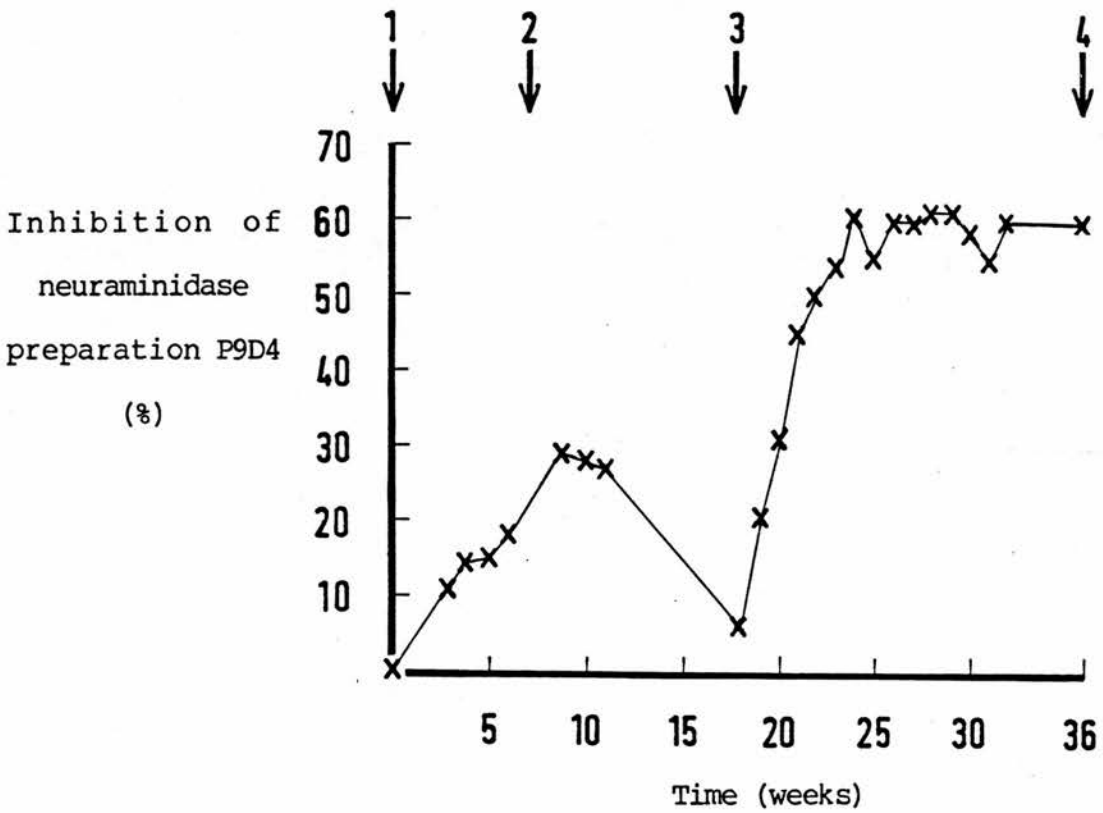


Fig. VI/9 - Immunisation of Rabbit 4 with Clostridium perfringens Sigma neuraminidase (see text); the arrows indicate antigen injections. Anti-neuraminidase response measured as inhibition of C. perfringens neuraminidase preparation P9D4 by test serum samples diluted 1 in 100 before assay.

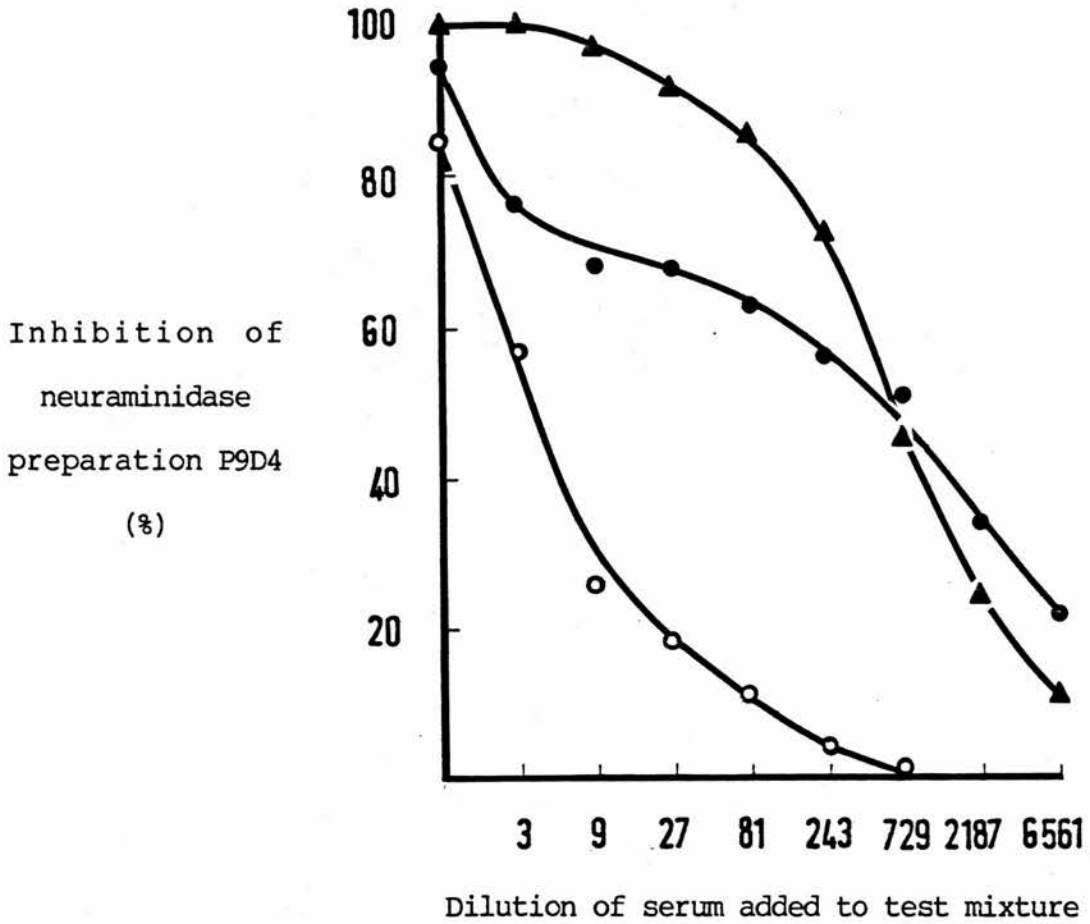


Fig. VI/10 - Inhibition of Clostridium perfringens neuraminidase by serial dilutions of three antisera: ●—●, Rabbit-4 weeks 23-32 pooled serum; o—o, Rabbit-4 pre-inoculation serum; ▲—▲, C. perfringens 1863 serum (see text).

a better secondary response may occur if the primary response is allowed to decline before further antigen administration.

The titration curves shown in fig. VI/11 were performed with a similar amount of Sigma neuraminidase as test enzyme in place of the standard C. perfringens strain L2Ab preparation. Comparison of figs VI/10 and 11 shows that, although the values obtained for the equine serum 1863 were very similar, the Rabbit-4 serum did appear rather more active in assays with the Sigma preparation as test enzyme. It seems that our standard assays with P9D4 enzyme give reliable results with the equine antisera but may slightly underestimate the anti-neuraminidase content in sera from rabbits immunised with the Sigma enzyme. This possibility was not further investigated since the discrepancy was not great and our estimates are on the conservative side. Furthermore, assays with the C. perfringens strain L2Ab enzyme may be more valid for the assessment of the protective potency of antisera for animals challenged with the L2Ab strain (see Section VIIe).

Rabbit 5 was immunised with larger doses of the Sigma neuraminidase preparation (see table VI/IV and fig. VI/12). The initial subcutaneous injection contained 5.0 units of neuraminidase; its protein content was low (0.8 mg) because this batch of enzyme had a slightly higher specific activity than the previous batches (see Materials and Methods). There was again a prolonged primary response; the inhibitory level rose steadily to c. 50% during the first five weeks and thereafter remained around this level although no further injections were given during the next six months. A second dose of antigen, with aluminium

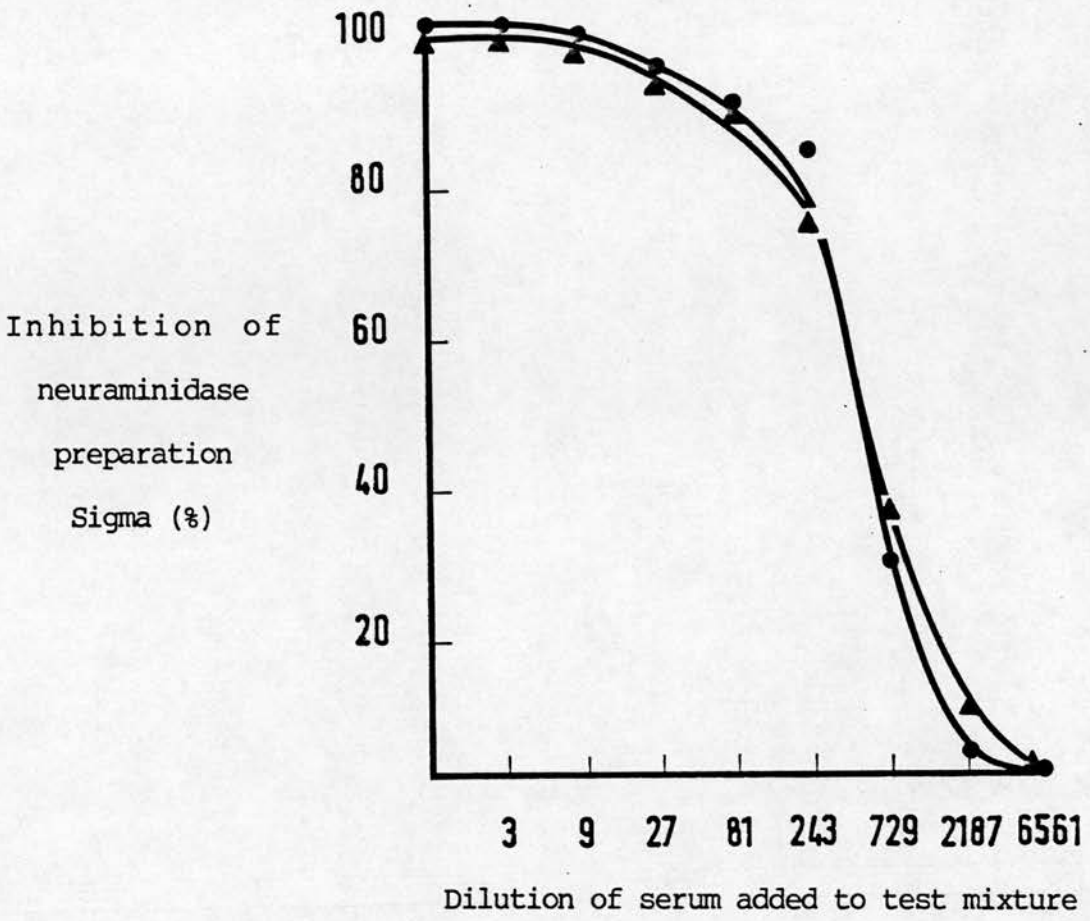


Fig. VI/11 - Inhibition of Clostridium perfringens Sigma neuraminidase by serial dilutions of two antisera: ●—●, Rabbit-4 week-32 serum; ▲—▲, C. perfringens 1863 serum (see text). The reference assay with the test dose of Sigma neuraminidase in the absence of serum gave a value (E_{549}) of 0.681 after incubation with substrate FVII(8) for 30 min.

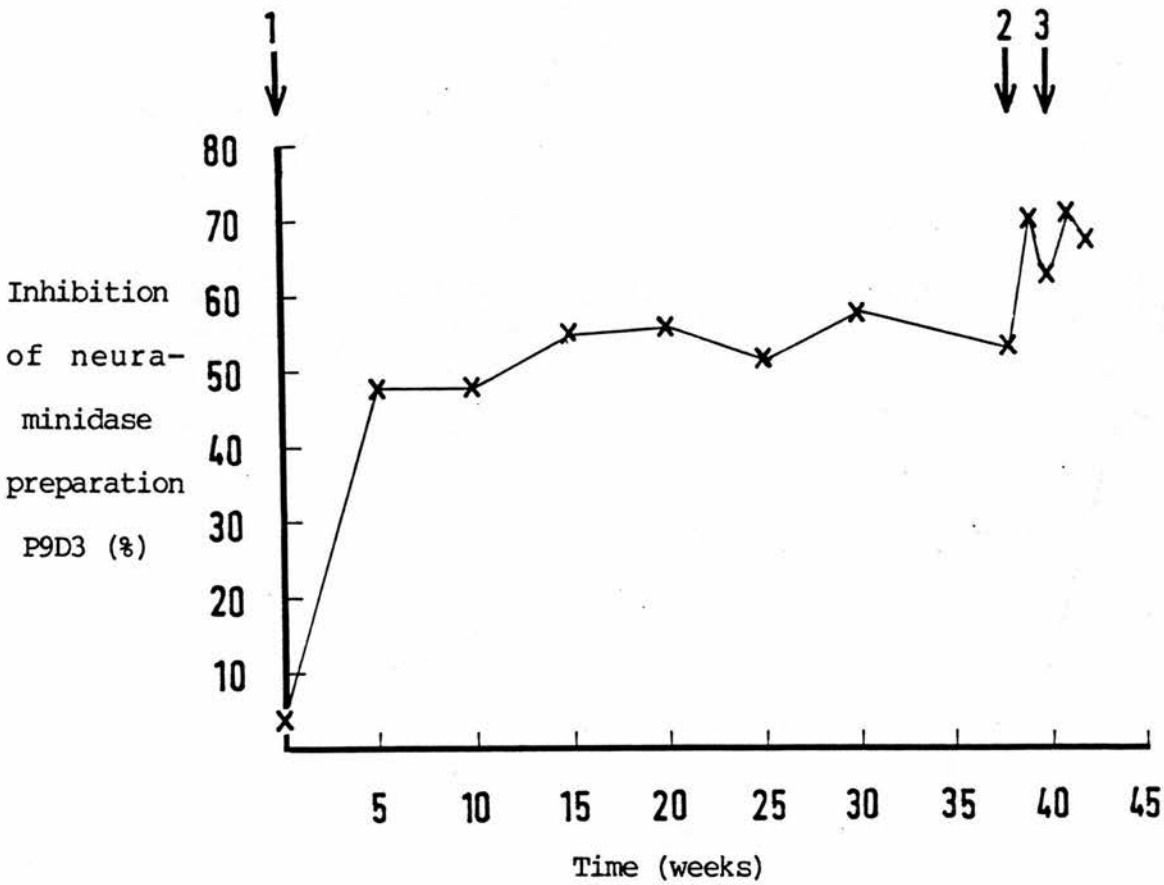


Fig. VI/12 - Immunisation of Rabbit 5 with Clostridium perfringens Sigma neuraminidase (see text); the arrows indicate antigen injections. Anti-neuraminidase response measured as inhibition of C. perfringens neuraminidase preparation P9D3 by test serum samples diluted 1 in 100 before assay.

hydroxide as adjuvant, was given by intraperitoneal injection after 38 weeks and this boosted the inhibitory level to c. 70%. A further injection given two weeks later by the intravenous route did not further enhance the response. Since it was difficult to be certain whether each of these injections produced a short-lived rise in antibody that would rapidly fall again, the animal was killed at week 42 while the serum still had a high level of activity.

The titration curve obtained with the Rabbit-5 week-41 serum (fig. VI/13) shows that the inhibitory activity in this serum is very similar to that in the experimental equine 1863 serum; although it may give marginally less inhibition in high concentrations it appears to give better inhibition when diluted beyond c. 1 in 100. Comparison with the pre-inoculation serum confirms the production of specific anti-neuraminidase activity during the immunisation procedure.

Our experience in attempting to produce high-titre antisera by injecting C. perfringens neuraminidase into rabbits suggests that it is not a good antigen and that it is difficult to produce antibody that will completely inhibit the activity of the enzyme. The Rabbit-5 serum is the best that was obtained; its activity is very similar to that found in the experimental equine serum 1863. Further comparisons of these sera are included below.

VId. Evaluation of anti-neuraminidase and anti-phospholipase-C activities of selected sera

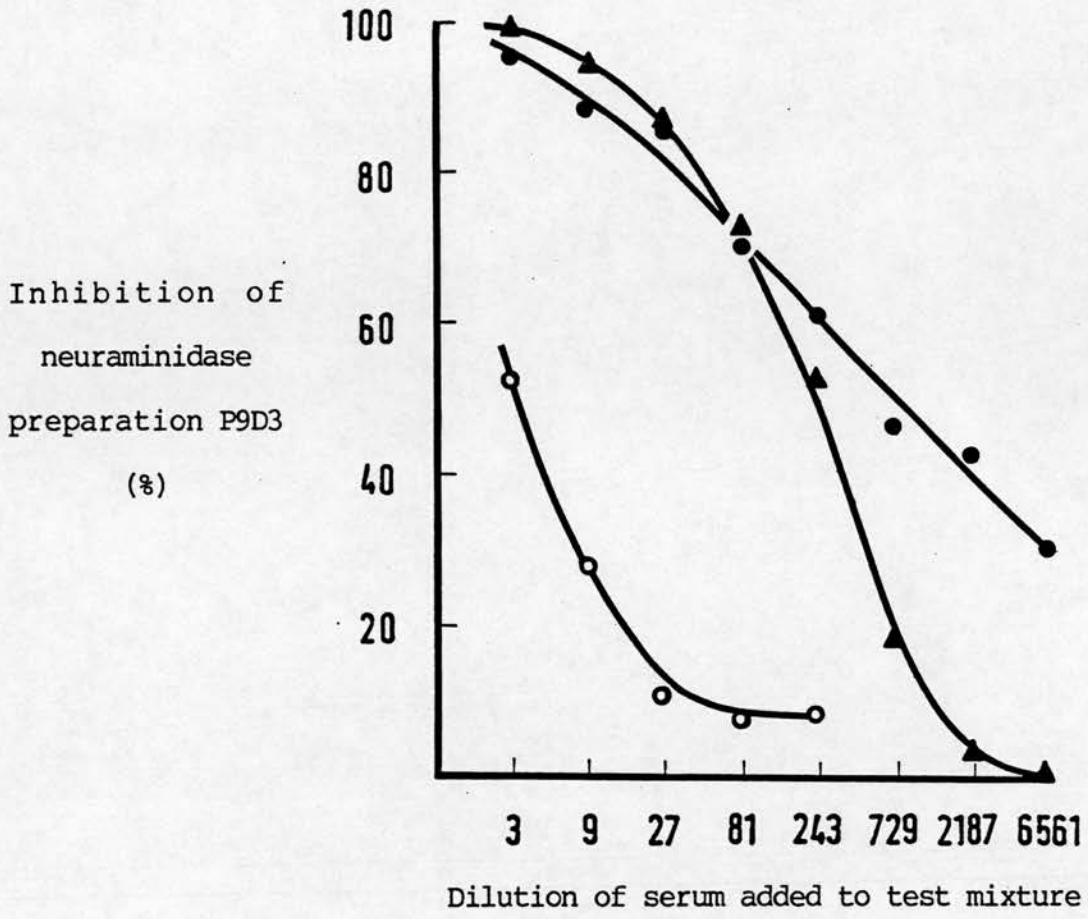


Fig. VI/13 - Inhibition of Clostridium perfringens neuraminidase by serial dilutions of three antisera: ●—●, Rabbit-5 week-41 serum; o—o, Rabbit-5 pre-inoculation serum; ▲—▲, C. perfringens 1863 serum (see text).

Table VI/V gives details of the antibody content of a number of sera that were tested for their ability to protect guinea pigs from experimental C. perfringens infection in later studies (see Section VIIe). The equine antisera are described in Materials and Methods. The rabbit sera tested were the pre-inoculation sera from Rabbits 3, 4 and 5 (R3P, R4P and R5P), the pooled weeks 8-11 sample from Rabbit 3, the pooled weeks 23-32 sample from Rabbit 4 and the week-41 sample from Rabbit 5 (see Section VIc). Anti-neuraminidase assays were performed at the standard dilution of 1 in 100 as previously. The anti-phospholipase-C (anti-α-toxin) content was titrated as described in Materials and Methods with a test enzyme preparation (PLC) prepared from the supernate of a culture of C. perfringens strain L2Ab in trypticase glycogen medium (TGM).

Serum CPA2, a second batch of the commercially available C. perfringens type A diagnostic antiserum, contained moderate anti-neuraminidase activity (comparable to that of the first batch, CPA1; see fig. VI/2) as well as the anticipated potent anti-phospholipase activity. The experimental equine antisera prepared against C. perfringens culture products contained varying amounts of the two antibodies. The 1863 serum had the highest anti-neuraminidase activity and also had a high anti-phospholipase content. Sera 2748 and 1881 had lower levels of anti-neuraminidase but both had strong anti-phospholipase activity. The 5438 serum had very low levels of antibodies, as did the C. tetani ATS serum. The values for anti-neuraminidase activity in the rabbit antisera were similar to those obtained previously (figs VI/7, 9 and 12); none had detectable anti-phospholipase activity. Although each of these assays gives an indication of the relative amounts of

TABLE VI/V

The relative anti-neuraminidase and anti-phospholipase-C activities of various sera used in guinea pig protection tests

Serum[a]	Anti-neuraminidase activity[b]	Anti-phospholipase titre[c]
Equine		
CPA2	60	20,000
1863	85	20,000
1881	19	10,000
2748	59	20,000
5438	6	4
ATS	1	40
Rabbit		
R3P	1	<2
R3(8-11)	58	<2
R4P	5	<2
R4(23-32)	60	<2
R5P	8	<2
R5(41)	69	<2

[a] See text.

[b] Anti-neuraminidase activity measured as inhibition of test enzyme preparation P9D4 with sera diluted 1 in 100.

[c] Anti-phospholipase titre is the reciprocal of the highest dilution of serum that inhibited the activity of the test enzyme preparation PLC.

inhibitory activity for that enzyme in different sera, it should be appreciated that the relative sensitivity of the two assays for inhibition of the different enzymes is unknown. Thus, although serum 1863 had high activity against each enzyme, it seems probable that the amount of anti-phospholipase antibody (sufficient to inhibit the test enzyme with the serum diluted 1 in 20,000) is considerably greater than the amount of anti-neuraminidase (sufficient to give 85% inhibition of the test enzyme with the serum diluted 1 in 100).

VII. PATHOGENICITY AND NEURAMINIDASE PRODUCTION OF CLOSTRIDIA IN GUINEA PIGS

VIIa. Virulence of C. perfringens strain L2Ab in experimental guinea-pig infection

The virulence of the classical C. perfringens strain L2Ab was tested by injecting graded numbers of washed log-phase cells into the muscle mass of the left hind leg in a series of guinea pigs (see Materials and Methods). Table VII/I gives the results of one such experiment; the sclerosing agent $CaCl_2$ was injected into the same site 3 h previously in order to promote infection. After microscopic estimation of the total count of organisms in the suspension of washed cells, a sample was diluted in sterile saline so that it contained 2×10^5 cells/ml (i.e. 4×10^4 cells in the 0.2-ml inoculum given to the first animal); further 10-fold dilutions were made for the other inocula. Viable counts made with this series of dilutions gave results very close to those for total cell counts (see Materials and Methods).

Animals given challenge doses of 4×10^2 or more cells developed acute overwhelming infection and died or were killed within 24 h; the animal given c. 40 organisms was also severely ill after 24 h and died during the second day. All these animals showed the classical appearances of experimental gangrene (G in table VII/I, see Materials and Methods for classification system); the inoculated limb was markedly swollen and post-mortem examination revealed extensive necrosis and dissolution of the muscle mass with haemorrhagic oedematous exudate spreading up over

TABLE VII/I

Virulence of Clostridium perfringens strain L2Ab for guinea pigs, tested with $CaCl_2$ as initiating agent

Challenge dose[a] (no. of organisms)	Outcome of challenge[b]
4×10^4	G; dead by day 1
4×10^3	G; killed on day 1[c]
4×10^2	G; dead by day 1
4×10^1	G; dead by day 2
4	MA; killed on day 7
4	MA; killed on day 7
Nil	Slight local swelling; NAD when killed on day 7

[a] Total count of organisms in inoculum (0.2 ml) to left hind thigh; the control animal received sterile saline with no bacteria. $CaCl_2$ was injected to the same site 3 h previously in all animals.

[b] See Materials and Methods for classification of outcome of challenge; further details in text.

[c] Animal killed because of severe infection.

the anterior abdominal wall. C. perfringens with culture characteristics typical of strain L2Ab was reisolated from the infected tissue. All samples were shown to contain many characteristic large Gram-positive bacilli in direct Gram films of impression smears; there were very few polymorphonuclear leucocytes present.

Two animals given an inoculum containing only c. 4 cells survived and did not develop gangrene. In these animals infection was established but it was contained locally; they developed stiffness and swelling in the inoculated leg during the first 2-3 days but this did not increase further and the animals remained lively. At post-mortem examination there was a medium-sized abscess (MA) localised in the muscle of the inoculated thigh. Gram films showed many polymorphonuclear cells and smaller numbers of Gram-positive bacilli, some of which were degenerate in appearance (beaded or becoming Gram-negative); some intracellular Gram-positive material was also seen. Typical C. perfringens were reisolated.

Control animals, given $CaCl_2$ but no bacterial inoculum, were included with each batch of test animals during these studies. In some cases, as in this experiment, the animal developed a limited degree of stiffness and swelling in the inoculated limb over the first two days but this tended to improve thereafter and no abnormality was seen at post-mortem inspection of the inoculated muscle. On other occasions, however, the injection of $CaCl_2$ produced a moderate degree of swelling and a small localised abscess (SA) was found when the animal was killed (e.g. tables VII/III, VI and VII). Microscopy and culture failed to reveal the presence of organisms

in these sterile necrotic foci, which contained only small numbers of inflammatory cells. There was no obvious explanation for the different response to $CaCl_2$ in different animals; it did not appear to be related to the weight of the test animal, but it might be attributable to inevitable minor variation in the exact site of inoculation.

A similar experiment was performed but with adrenaline as initiating agent instead of $CaCl_2$; this produces an area of transient vasoconstriction and ischaemia that may permit establishment of infection. Table VII/II gives the results when the inocula contained graded challenge doses of the same strain of C. perfringens mixed with adrenaline (see Materials and Methods). In this experiment two animals were inoculated at each challenge dose; they were observed for only four days and no tissue samples were taken. The animals given 10^7 cells developed classical gangrene and died within 24 h; those given 10^6 cells also developed gangrene and were killed at 24 h. Animals that received 10^5 or 10^4 cells developed only slight transient signs in the left leg but had apparently recovered fully by the second or third day, while those given less than 10^4 cells showed no obvious ill effects.

It is apparent that C. perfringens strain L2Ab is a virulent strain capable of producing experimental gangrene in guinea pigs. A considerably higher challenge dose was required to establish infection with adrenaline, which produces a transient ischaemic focus, than with $CaCl_2$, a sclerosing agent that may produce a small necrotic focus even in control animals. No attempt was made to determine accurately the lethal dose (LD50) of the organism in these models of infection but the results of these experiments with

TABLE VII/II

Virulence of Clostridium perfringens strain L2Ab for guinea pigs, tested with adrenaline as initiating agent

Challenge dose[a] (no. of organisms)	Outcome of challenge[b]
10^7	G; dead by day 1
10^6	G; killed on day 1[c]
10^5	Transient swelling days 1-2
10^4	Transient swelling day 1
10^3	Nil
10^2	Nil
10^1	Nil
Nil	Nil

[a] See footnote to table VII/I; animals given adrenaline (10 µg) instead of $CaCl_2$ as initiating agent (see Materials and Methods).

[b] See footnote to table VII/I. Two animals were injected at each challenge dose, with similar outcome; animals were observed for 4 days.

[c] Animals killed because of severe infection.

very small numbers of animals proved reasonably reproducible. The observed minimum lethal doses in these experiments (between 4 and 40 cells with $CaCl_2$, and between 10^5 and 10^6 cells with adrenaline) were consistent with those found in repeat experiments (see Sections VIIb and e), and similar experiments with graded doses of other strains of C. perfringens also usually gave a clear-cut endpoint (see Section VIIc).

VIIb. Production of neuraminidase during infection with C. perfringens strain L2Ab

Tissue samples taken at post-mortem examination of various animals challenged with C. perfringens strain L2Ab were homogenised and tested for neuraminidase activity (see Materials and Methods). Table VII/III gives the results of an experiment similar to that recorded in table VII/I, with graded doses of the organism inoculated into guinea pigs after $CaCl_2$ injection. The animals receiving inocula of 10^3 or 10^2 cells developed classical gangrene and died between 24 and 48 h after challenge; the animal given c. 10 cells survived but developed a localised infection with a small abscess in the inoculated muscle. The minimum lethal dose in this experiment (between 10 and 100 cells) was comparable to that found in the previous experiment (between 4 and 40 cells; table VII/I).

Neuraminidase was present in large amounts in the tissue samples taken from the inoculated limbs of the two animals that developed classical gangrene and died within 48 h; it was confirmed that the assay product had the typical absorption

TABLE VII/III

Production of neuraminidase during infection of guinea pigs by Clostridium perfringens strain L2Ab, with $CaCl_2$ as initiating agent

Challenge dose[a] (no. of organisms)	Outcome of challenge[a]	Relative amount of neuraminidase[b] in tissue sample from		
		L. leg	R. leg	Liver
10^3	G; dead by day 2	+++	+++	+
10^2	G; dead by day 2	+++	++	±
10^1	SA; killed on day 6	++	±	-
Nil	SA; killed on day 6	-	-	-

[a] See footnotes to table VII/I; the results for a control animal that developed a sterile abscess after $CaCl_2$ injection in a separate experiment are included for comparison.

[b] See Materials and Methods for grading of neuraminidase activity in homogenised tissue samples.

spectrum for NANA, with peak absorption at 549 nm. Moderate amounts of enzyme were found in the samples from the uninoculated leg in these animals and there was also some activity in the liver samples. This enzyme might have been blood-borne or due to septicaemic spread of the organism, but it might also have been due to direct extension of the original lesion from the left thigh to involve the lower abdominal wall and the opposite limb; some spread might have occurred post mortem, as these animals died during the night and had been dead for some time before dissection next morning. It is also possible that there was some superficial contamination of the samples with the enzyme-containing oedema fluid that tended to overflow from the infected areas during the sampling procedure.

Enzyme activity (score ++) was also clearly shown in the sample of muscle containing a small localised abscess following the administration of a very low challenge dose. By contrast, even with assays incubated for 24 h, no neuraminidase activity could be demonstrated in the sterile localised abscess of comparable size produced in a control animal by injection of $CaCl_2$ but no bacteria. Both of these samples were taken from freshly killed animals 6 days after inoculation. Assays for neuraminidase in the right leg and liver of the control animal were also completely negative.

Table VII/IV gives the results of an equivalent experiment when the same strain of C. perfringens was inoculated into guinea pigs with 10 μg adrenaline as initiating agent. On this occasion the animal given 10^7 cells developed gangrene and died within 24 h; the animals given 10^6 or 10^5 cells developed very extensive necrotic infection of the inoculated limb although neither died

TABLE VII/IV

Production of neuraminidase during infection of guinea pigs by Clostridium perfringens strain L2Ab, with adrenaline as initiating agent

Challenge dose[a] (no. of organisms)	Outcome of challenge[b]	Relative amount of neuraminidase[c] in tissue sample from		
		L. leg	R. leg	Liver
10^7	G; dead by day 1	+++	...	±
10^6	N; killed on day 3	+++	±	-
10^5	N; killed on day 3	+++	+	-

[a] See footnote to table VII/II.

[b] See footnote to table VII/I.

[c] See footnote to table VII/III; ... = not tested.

within 3 days. Neuraminidase was present in large amounts in the infected limbs, and smaller amounts were again detected in some of the other tissue samples.

These results are characteristic of those found in a number of similar experiments. It is apparent that neuraminidase is produced in the tissues during infection by C. perfringens strain L2Ab, whether in animals developing a full-blown gangrenous infection or in those in which a low challenge dose produces only a small abscess that is localised to the area where $CaCl_2$ was injected. Our test procedures, however, do not detect any neuraminidase activity in normal guinea-pig muscle or liver, nor in muscle containing a sterile abscess produced by injection of $CaCl_2$ alone.

VIIc. Pathogenicity and neuraminidase production of other strains of C. perfringens type A in guinea pigs

A range of strains of C. perfringens type A that produce different amounts of neuraminidase and of phospholipase-C (α-toxin) in vitro were assessed for their ability to produce infection in guinea pigs by a procedure similar to that used with strain L2Ab. Large challenge doses (c. 10^8 cells) were inoculated into the left hind leg 3 h after an injection of $CaCl_2$ into the same site. The results of several batches of tests have been amalgamated in table VII/V, where the strains are subdivided according to their ability to produce neuraminidase in vitro; within each group they are roughly ranked according to their pathogenicity in these tests.

TABLE VII/V

Pathogenicity and production of neuraminidase with large inocula of strains of Clostridium perfringens type A injected into guinea pigs with $CaCl_2$ as initiating agent

Strain number[a]	Challenge dose (no. of organisms)	Outcome of challenge[b]	Relative amount of neuraminidase in L. leg tissue sample[b]
Cl	1.7×10^8	G; dead by day 1	+++
Hobbs' xvi	1.9×10^8	G; dead by day 1	...
Hobbs' xviii	1.1×10^8	G; killed on day 1	+++
Hobbs' xvii	2.5×10^8	G; killed on day 1	...
Hobbs' i	1.3×10^8	G; killed on day 2	...
CW6	1.9×10^8	N; killed on day 3	+++
CW7	2.0×10^8	LA; killed on day 7	+++
Hobbs' x	2.0×10^8	LA; killed on day 7	...
NCTC11144	1.2×10^8	MA; killed on day 6	+++
Hobbs' vi	2.4×10^8	MA; killed on day 7	...
Hobbs' ix	1.4×10^8	MA; killed on day 7	...
NCTC10240[c]	1.0×10^8	MA; killed on day 6	...
Hobbs' 18	3.8×10^8	MA; killed on day 7	...
Hobbs' 24	3.8×10^8	MA; killed on day 7	...
Hobbs' viii	1.1×10^8	G; killed on day 2	-
Hobbs' 21	1.1×10^8	LA; killed on day 7	...
Hobbs' xiii	2.6×10^7	MA; killed on day 7	-
Hobbs' xv	8.0×10^7	MA; killed on day 6	-
Hobbs' 14	2.5×10^8	MA; killed on day 7	-
Hobbs' 2	1.8×10^8	SA; killed on day 7	-
4621[c]	2.3×10^8	SA; killed on day 7	-

[a] Strains that produce neuraminidase *in vitro* in upper part of table; neuraminidase-negative strains below (see tables III/II, V and XIII).

[b] See footnotes to table VII/III; ... = not tested.

[c] Both strains of Hobbs' serotype 13.

The classical strain Cl and the food-poisoning Hobbs' type-xviii strain both caused gangrene in the test animals and it was confirmed that large amounts of neuraminidase were present in the infected tissues. The strains of Hobbs' serotypes xvi and xvii also caused gangrenous infection. The Hobbs' type-i strain produced only slightly less dramatic illness and when the animal was killed at 48 h there was extensive muscle necrosis (but see below for further tests; table VII/VII). These food-poisoning strains are all similar to classical strains in that they are haemolytic and produce relatively heat-sensitive spores; none of the "typical food-poisoning" strains (non-haemolytic, heat-resistant) produced gangrenous infection in these studies.

Strain CW6, a strain that does not produce phospholipase-C, did not cause the full picture of gangrene but produced extensive destruction of the inoculated muscle; the animal became unwell and was killed on day 3. Strain CW7, also phospholipase-negative, was less virulent but produced a large localised abscess in the inoculated muscle. Strain NCTC11144 is also neuraminidase-positive and phospholipase-negative; it produced a smaller localised abscess. Large amounts of neuraminidase were demonstrated in the tissues from these three animals. A number of other neuraminidase-positive strains were tested and found to produce only localised infection.

Of the neuraminidase-negative organisms, only the Hobbs' type-viii strain produced acute gangrenous infection; the other strains tested produced localised abscesses. No neuraminidase was demonstrable in the tissue samples from these animals, regardless of the severity of infection produced.

Further tests were performed in order to amplify and confirm these findings with a number of the strains that produced gangrenous infection at high challenge doses in the initial series of tests. Table VII/VI gives the results when graded doses of the strain of Hobbs' serotype xviii were injected into guinea pigs with $CaCl_2$ as initiating agent. It was confirmed that this strain could produce classical gangrenous infection; it proved less virulent than strain L2Ab (see table VII/I), having a minimum lethal dose between 4×10^3 and 4×10^4 organisms. When similar tests were performed with the Hobbs' type-i strain, it proved less virulent. In the initial test (table VII/V) an inoculum of 1.3×10^8 cells produced gangrenous infection; the animal was judged to be severely ill and was killed at 48 h. However, in the repeat tests with graded challenge doses of this strain (table VII/VII) only localised infections were produced; even at the highest challenge dose tested (6×10^7 cells) the animal survived, although the limb was very swollen and the muscle mass was found to have been almost entirely destroyed. It appears that high challenge doses of this strain can cause very severe localised infection of the muscle but that the organism may not be able to produce the full classical picture of generalised "toxaemia" and rapid death.

Further tests were performed with the Hobbs' type-viii strain, the only neuraminidase-negative strain that was found to be virulent in the initial tests shown in table VII/V. An experiment was performed with graded challenge doses of this strain, following the same procedures as were used for assessing the virulence of strains L2Ab, Hobbs' xviii and Hobbs' i above. On this occasion, however, challenge doses of 1.3×10^5 - 1.3×10^8 organisms

TABLE VII/VI

Virulence of Clostridium perfringens strain Hobbs' xviii for guinea pigs, tested with $CaCl_2$ as initiating agent

Challenge dose[a] (no. of organisms)	Outcome of challenge[a]
4×10^7	G; killed on day 2
4×10^6	G; killed on day 2
4×10^5	...
4×10^4	G; dead by day 2
4×10^3	MA; killed on day 6
4×10^2	MA; killed on day 6
4×10^1	SA; killed on day 6
Nil	SA; killed on day 6

[a] See footnotes to table VII/I; ... = animal omitted from experiment because of error in inoculation.

TABLE VII/VII

Virulence of Clostridium perfringens strain Hobbs' i for guinea pigs, tested with $CaCl_2$ as initiating agent

Challenge dose[a] (no. of organisms)	Outcome of challenge[a]
6×10^7	N
6×10^6	LA
6×10^5	MA
6×10^4	SA
6×10^3	MA
6×10^2	SA
6×10^1	MA
6	SA
Nil	SA

[a] See footnotes to table VII/I; all animals killed on day 6.

produced only small or medium-sized localised abscesses (SA or MA) with no generalised illness; lower doses produced slight swelling and stiffness of the inoculated limb but no infection was demonstrated when the animals were killed after 6 days. It is not clear why the organisms proved less virulent in these tests than in the initial test. There was no unusual delay in preparing or inoculating the bacteria, and viable counts with the diluted inocula were very close to the total counts. On each occasion the organism had been freshly grown from a vial of the same freeze-dried stock, and on each occasion the organism reisolated from the infected limb was shown to have the typical properties of the Hobbs' type-viii strain, i.e. weak phospholipase activity inhibited by C. perfringens antitoxin on EYA plates, production of haemolysis on BA plates, no neuraminidase produced in THB cultures. The tissue sample from the infected limb of the animal given the highest challenge dose in this experiment was also tested and confirmed to be neuraminidase-negative.

Further tests were performed, inoculating guinea pigs with high challenge doses of the organisms that had been reisolated from the infected limbs in the first two experiments with the Hobbs' type-viii strain. Table VII/VIII summarises these experiments. Although the conflicting results of the first two experiments raised some doubts about the virulence of this strain, the results of challenge with the same strain reisolated from the original infections were quite convincing - all the animals developed classical gangrenous infection; three died within 24-48 h and the fourth became severely ill and was killed at 24 h. Typical organisms were again reisolated from the infected leg samples and

TABLE VII/VIII

Pathogenicity for guinea pigs of Clostridium perfringens strain Hobbs' viii before and after animal passage

Experiment number[a]	Challenge dose (no. of organisms)[b]	Outcome of challenge[b]
1	1.1×10^8	G; killed on day 2
2	1.3×10^8	MA; killed on day 6
3	3.8×10^7	G; dead by day 2
	3.8×10^7	G; killed on day 1
4	8.5×10^7	G; dead by day 1
	8.5×10^7	G; dead by day 1

[a] The challenge organisms for Expts 1 and 2 were freshly grown from freeze-dried stock; the organisms for Expt 3 were reisolated from the animal infected in Expt 1; the organisms for Expt 4 were reisolated from Expt 2 (see text). All animals were given $CaCl_2$ 3 h before bacterial challenge.

[b] See footnotes to table VII/I; the results for Expt 1 are from table VII/V.

no neuraminidase was demonstrable in the tissue samples from the infected leg in any of these four animals, even in assays incubated for 24 h. It is possible that the virulence of the original stock was increased during animal passage; however, it is clear that this was not accompanied by any change in its neuraminidase-negative status.

These tests revealed a broad spectrum of pathogenicity for strains of C. perfringens type A in this animal model, where the injection of $CaCl_2$ provides a necrotic focus in which infection can be easily established. Very virulent strains, e.g. L2Ab, can produce the full classical picture of gangrene and rapid death even at very low challenge doses (10^1-10^2 cells); slightly less virulent strains, e.g. Hobbs' type xviii, produce the same picture but only at higher doses (10^4-10^5); some strains, e.g. Hobbs' type i, can produce severe local necrosis when large numbers (c. 10^8) are injected, but may not kill the animal; even large numbers of other strains of lower virulence produce only an abscess of greater or lesser size localised in the muscle around the site of injection of $CaCl_2$. Small numbers of virulent strains, or large numbers of low-virulence strains, are often able to establish a local infection and multiply to some extent; neuraminidase-positive strains can be shown to have produced the enzyme in vivo in this situation also.

Microscopic examination of Gram films of impression smears taken from the infected tissues of these animals gave results similar to those found with C. perfringens strain L2Ab. When animals developed acute gangrenous infection and died in 24-48 h the films contained tissue debris and many large Gram-positive

bacilli but only occasional polymorphonuclear leucocytes. When animals survived for 6 or 7 days with infection localised to the site of $CaCl_2$ inoculation the films contained many pus cells and smaller numbers of degenerate bacilli.

No clear correlation appears between virulence and production of either phospholipase-C (α-toxin) or neuraminidase in these studies (table VII/IX). Although the most virulent strains all produced phospholipase-C, many other phospholipase-producing strains were of low virulence and at least one phospholipase-negative strain (CW6) could produce an extensive necrotic infection in response to a large challenge dose. Similarly, although most of the virulent strains produced neuraminidase well in vitro, the neuraminidase-negative Hobbs' type-viii strain was undoubtedly fully pathogenic in this model of infection.

VIId. Pathogenicity and neuraminidase production of strains of other clostridial species in guinea pigs

The pathogenicity of a number of other species of Clostridium was investigated by the same procedures that were used for testing C. perfringens strains; high challenge doses of various neuraminidase-positive and neuraminidase-negative species were inoculated into the thigh muscles of guinea pigs that had already received an injection of $CaCl_2$ in the same site. In all cases, the challenge organism was recultured from the infected tissue after the death of the animal and the identity of the reisolate was confirmed by checking its cultural characteristics on BA plates and half-antitoxin EYA plates.

TABLE VII/IX

Correlation of pathogenicity and production of extracellular products for various strains of Clostridium perfringens type A

Strain number	Patho-genicity[a]	In-vitro production of		
		Phospholipase-C[b] (α-toxin)	Neuraminidase[c]	Haemolysin[d] (θ-toxin)
L2Ab	G	+	+	+
C1	G	+	+	+
xvi	G	+	+	+
xviii	G	+	+	+
viii	G	±	−	+
xvii	G	±	+	+
i	G/N	+	+	−
CW6	N	−	+	+
x	LA	+	+	+
21	LA	+	+	−
CW7	LA	−	+	+
vi	MA	+	+	+
13(10240)	MA	+	+	−
ix	MA	+	±	−
24	MA	+	±	−
xiii	MA	+	−	−
xv	MA	+	−	−
14	MA	±	−	−
NCTC11144	MA	−	+	+
18	MA	±	+	−
2	SA	+	−	−
13(4621)	SA	+	−	−

[a] Test strains ranked in approximate descending order of virulence; for fuller details of outcome of inoculation with high challenge doses for each organism, see tables VII/I, V, VII and VIII.

[b] For phospholipase-C production, see Section IIId.

[c] For neuraminidase production, see tables III/II, IV, V, VI and XIII. The consistently small amounts of enzyme produced by the Hobbs' type-24 strain is recorded here as ±.

[d] For haemolysin production, see table III/V and Section IIId.

In table VII/X, both total and viable counts of the number of organisms in the challenge dose are given. There was very little loss of viability in the washed cell suspensions of C. absonum and C. bifermentans, and this was also true for a number of strains of C. perfringens that were included in the same batches of experiments. There was, however, a reduction in count by a factor of 10^1-10^2 with C. septicum, C. paraperfringens and many of the C. sordelli strains; the fall was of the order of 10^3 with C. novyi and C. sordelli strain P3. It is likely that these strains are more delicate (sensitive to Eh changes, cooling, suspension in saline, etc.) than C. perfringens and that the total count gives an overestimate of the effective challenge dose with these organisms. Viable spore counts were also performed with pasteurised samples of the washed cell suspensions (see Materials and Methods). Very few resistant spores were present in these log-phase preparations with C. perfringens, C absonum, C. paraperfringens and most of the C. sordelli strains. However, counts of the order of 10^2-10^3 spores were obtained in the inoculum (0.2 ml) of the strains of C. novyi and C. bifermentans and even higher counts (10^4-10^5) with C. septicum and C. sordelli strain 1734. Thus, although the challenge dose was predominantly in the form of vegetative cells for most strains tested, spores may have formed a substantial proportion of the inoculum with C. novyi, C. septicum and C. sordelli strain 1734.

The strains of C. septicum and C. novyi type A each killed the test animal within 24 h, producing overwhelming gangrenous infection similar to that seen with C. perfringens strain L2Ab, with marked swelling, extensive haemorrhagic oedema and necrosis

TABLE VII/X

Pathogenicity and production of neuraminidase with large inocula of various clostridial species injected into guinea pigs with $CaCl_2$ as initiating agent

Organism and strain number[a]	Challenge dose[b] (no. of organisms) Total count	Viable count	Outcome of challenge[c]	Relative amount of neuraminidase in L. leg tissue sample[d]
C. septicum				
NCTC547	1.2×10^6	1.2×10^5	G; dead by day 1	+++
C. sordelli				
1734	7.5×10^7	1.2×10^6	S; dead by day 2	++
1734 (repeat)	9.8×10^7	6.6×10^5	S; dead by day 1	++
CB2	2.0×10^7	1.2×10^6	LA; dead by day 3	±
P3	5.8×10^7	4.0×10^4	LA; killed on day 5	+
CB3	1.4×10^8	6.0×10^6	MA; killed on day 6	±
CB4	4.4×10^7	1.8×10^7	MA; killed on day 6	±
C. absonum				
HA7103	4.5×10^7	2.7×10^7	MA; killed on day 6	+++
HA7107	1.4×10^8	7.6×10^7	SA; killed on day 6	++
C. novyi type A				
NCTC538	3.1×10^6	2.0×10^3	G; dead by day 1	-
C. bifermentans				
B4	6.1×10^7	4.4×10^7	SA; killed on day 6	-
C. paraperfringens				
3-3	1.4×10^7	8.8×10^5	SA; killed on day 6	-
2227	7.9×10^7	3.0×10^5	SA; killed on day 6	-

[a] Strains that produce neuraminidase *in vitro* in upper part of table; neuraminidase-negative strains below (see tables IV/I, II, V and VII).

[b] See Materials and Methods for counting methods.

[c] See footnote to table VII/I; S = muscle very swollen but no abscess or oedema.

[d] See footnote to table VII/III.

of the inoculated muscle. In each case microscopy showed many Gram-positive bacilli but very few pus cells. Neuraminidase was clearly shown to have been produced during the C. septicum infection, but no enzyme was detectable during the equally severe infection produced by the neuraminidase-negative species, C. novyi. C. absonum and C. paraperfringens are non-pathogenic species that are similar to C. perfringens but with differences in their phospholipase-C (see Materials and Methods); they proved to be of very low virulence in these tests, producing only small localised abscesses around the site of $CaCl_2$ inoculation. However, here too, the neuraminidase-positive C. absonum strains were shown to produce the enzyme in vivo while the non-neuraminidase-producing C. paraperfringens did not.

The strains of C. sordelli proved to be of varied virulence. On two occasions the inoculation of strain 1734 killed the test animal within 48 h. The appearances at post-mortem examination differed from those with the other gas-gangrene species; there was extensive gelatinous exudate spreading over the surface of the inoculated thigh muscles but no necrosis of the muscle itself, which was swollen, firm and pink. Microscopy showed many Gram-positive bacilli, some containing spores, but few pus cells. C. sordelli strain CB2 also killed the test animal, but after 3 days; there was a large localised necrotic abscess in the inoculated thigh, similar to that seen with a number of strains of C. perfringens (see table VII/V). Strain P3 was a little less virulent; the animal also developed a large localised abscess but survived for 5 days. The two other C. sordelli strains produced

localised abscesses but did not kill the test animals; these reactions were little more marked than that found with the non-pathogenic C. bifermentans strain.

These strains were further tested in order to determine which of them produced the C. sordelli β-toxin that is held to be responsible for the pathogenicity of this species (Willis, 1969, p. 226). When samples of CMB culture supernate were tested in guinea-pig skin tests (see Materials and Methods), only strain 1734 produced the purple-brown necrotic response typical for C. sordelli β-toxin; there was no response after injection of sterile CMB supernate or the supernates of cultures of C. sordelli strains CB2, CB3, CB4, P3 or C. bifermentans strain B4. This correlates with our finding that only strain 1734 produced the appearances typical of pathogenic C. sordelli on intramuscular injection.

There may be a rough correlation between virulence and the ability to produce neuraminidase in vitro for these strains (see table IV/II); C. sordelli strains 1734, CB2 and P3 produced larger amounts of the enzyme in PPW5 cultures (score +++) than strains CB3 (++) or CB4 (+), while C. bifermentans strains produced none. However, it should be borne in mind that this quantitation is rather imprecise in view of the marked variations found on different occasions in the amounts of enzyme produced by various species in repeat cultures in the same medium, or in other media (c.f. scores in THB medium, table IV/II). No enzyme activity could be detected in the tissues of the animal inoculated with C. bifermentans but the enzyme was clearly shown to be produced in vivo in moderate amounts by strain 1734 and in lesser amounts by the other strains of C. sordelli (table VII/X). There may again

appear to be a rough correlation with the virulence of these strains, but this should not be overstated because of the unstandard nature of the tissue samples in different types of infection and after different periods of time (see Materials and Methods).

These experiments bear out the conclusions from the previous tests with a range of C. perfringens strains. In this model, high challenge doses of many clostridial species can produce at least a limited degree of local infection in the necrotic focus produced by $CaCl_2$. During such infection, neuraminidase is produced in vivo by strains that have been found to produce it in vitro; no strain previously classified as neuraminidase-negative was induced to produce the enzyme in vivo. Although a number of pathogenic species produce the enzyme well, there is not a clear-cut correlation between enzyme production and virulence; in particular, the classical picture of overwhelming gangrenous infection is produced not only by neuraminidase-positive species, but also by the completely neuraminidase-negative C. novyi type A.

VIIe. Protective effect of various antisera for guinea pigs challenged with C. perfringens strain L2Ab

Table VII/XI summarises the results obtained when a selection of antisera were tested for their ability to protect guinea pigs challenged with C. perfringens strain L2Ab with adrenaline used as initiating agent. The antibody content of the sera tested was included in table VI/V above. Each animal was given antiserum by intraperitoneal injection 20 h before challenge with 10^6 cells (see

TABLE VII/XI

Protective effect of various antisera for guinea pigs challenged with Clostridium perfringens strain L2Ab with adrenaline as initiating agent

Antiserum given to animal[a]	Outcome of challenge[b]	Relative amount of neuraminidase in L. leg tissue sample[c]
Equine		
CPA2	NAD; killed on day 6	-
1863	SA; killed on day 6	...
1881	NAD; killed on day 6	±
2748	NAD; killed on day 6	-
5438	G; killed on day 1	+++
ATS	N; killed on day 3	+++
Rabbit		
R3P	G; dead by day 2	+++
R3(8-11)	G; dead by day 2	+++
Nil	N; killed on day 3	+++

[a] For antisera, see text. 1 ml of a 1 in 2 dilution of antiserum in sterile saline given by IP injection 20 h before challenge with 10^6 cells and 10 µg adrenaline as initiating agent; control animal given 1 ml saline in place of antiserum.

[b] See Materials and Methods for outcome of challenge; further details in text.

[c] See Materials and Methods for grading of neuraminidase activity in homogenised tissue samples; ... = specimen lost.

Materials and Methods); the challenge dose was the minimum lethal dose determined in previous experiments with this model of infection (see tables VII/II and IV). The control animal, given saline in place of antiserum, became seriously ill with extensive necrosis of the inoculated muscle and was killed on day 3. Animals given the equine sera CPA2, 1863, 1881 or 2748 were protected from infection; they developed only minor transient signs in the inoculated limb and little or no abnormality was seen when the animals were killed after 6 days. By contrast, animals that received the Rabbit-3 sera or the equine sera ATS or 5438 developed acute gangrenous infection similar to (or more severe than) that in the control animal. Assays for neuraminidase in tissue samples from the inoculated limbs showed that there was little or no enzyme present in the animals that were protected from infection whereas there were large amounts in the unprotected animals.

Similar results were obtained when the same antisera were tested in animals challenged with 10^2 cells with $CaCl_2$ used for initiation of infection (table VII/XII). The challenge dose, determined from previous experiments (see tables VII/I and III), produced an acute fatal infection in the control animal. The four equine antisera that gave protection when adrenaline was used for initiation of infection also gave protection in this experiment; infection was established in the necrotic focus produced by the injection of $CaCl_2$ but the animals survived with the infection localised in the inoculated leg. The equine ATS and 5438 sera gave no protection, the animals developing acute overwhelming infection. The results with the Rabbit-3 antisera were a little anomalous. The immune rabbit serum gave no protection but the animal given the

TABLE VII/XII

Protective effect of various antisera for guinea pigs challenged with Clostridium perfringens strain L2Ab with $CaCl_2$ as initiating agent

Antiserum given to animal[a]	Outcome of challenge[a]	Relative amount of neuraminidase in L. leg tissue sample[a]
Equine		
CPA2	MA; killed on day 6	+++
1863	SA; killed on day 6	+++
1881	MA; killed on day 6	+++
2748	MA; killed on day 6	+++
5438	G; dead by day 2	+++
ATS	G; killed on day 2	+++
Rabbit		
R3P	LA; killed on day 2	+++
R3(8-11)	G; dead by day 2	+++
Nil	G; dead by day 2	+++

[a] See footnotes to table VII/XI; animals challenged with 10^2 cells and given $CaCl_2$ in place of adrenaline as initiating agent.

pre-inoculation serum was less severely infected; when it was killed on day 2 the infection was localised to a large abscess at the site of inoculation. It is unlikely that this normal rabbit serum gave significant protection against infection in this experiment and this result was ascribed to the variation in response that should be expected with tests performed with single animals as in these studies. Although the result with the normal serum was anomalous there was no suggestion that the immune Rabbit-3 serum gave any protection with this model of infection. Large amounts of neuraminidase were present in all the samples of infected tissue; this confirmed our previous finding that the enzyme is produced when even minor degrees of infection are established (see Section VIIb).

The protective effect of this selection of sera was the same in both of the experimental models, i.e. with adrenaline and with $CaCl_2$, and appears to correlate with the content of anti-phospholipase-C (anti-α-toxin) rather than anti-neuraminidase (see table VI/V). The four protective equine sera all had high-titre anti-phospholipase activity whereas the two equine sera with very low levels were non-protective. In general the sera with high anti-phospholipase activity also had high anti-neuraminidase activity, but the 1881 serum had considerably lower anti-neuraminidase activity and was still clearly protective for the animals. The Rabbit-3 sera contained no anti-phospholipase activity and were non-protective; the anti-neuraminidase present in the immune serum had no apparent effect. Thus, although two models were established in which selected antisera could be shown

to give passive protection against challenge with C. perfringens type A, there was no evidence that the anti-neuraminidase activity in these sera might contribute to the protective effect.

A similar experiment was performed in order to assess the protective effect of the anti-neuraminidase sera raised in Rabbits 4 and 5 (table VII/XIII). Adrenaline was used to initiate infection and each serum was assessed at two challenge-dose levels - 10^6 and 10^7 cells of C. perfringens strain L2Ab. Previous experiments (tables VII/II, IV and XI) suggested that the minimum lethal dose for this strain injected with adrenaline was c. 10^6 cells; on this occasion even 10^5 cells produced typical acute gangrenous infection that killed the control animal within 24 h. Equine serum 1863 again proved to have a strong protective effect; at each challenge-dose level the animal developed slight local swelling but survived with only a small abscess localised at the site of inoculation. By contrast, none of the sera from Rabbits 4 and 5 gave protection against infection with strain L2Ab in these challenge doses; all the animals died with typical gangrenous infection in 1-2 days. Although the immune rabbit sera contained levels of anti-neuraminidase similar to that in the equine 1863 serum (see table VI/V and figs VI/10 and 13) they did not give protection and there is thus no evidence that the anti-neuraminidase content of serum 1863 contributes to its protective effect.

Figs VII/1 and 2 show the microscopic appearances in Gram films of material sampled from the lesions in two of the animals in this experiment. These appearances are also typical of those seen in the previous experiments with C. perfringens strains (Sections

TABLE VII/XIII

Protective effect of sera from Rabbits 4 and 5 for guinea pigs challenged with Clostridium perfringens strain L2Ab with adrenaline as initiating agent

Antiserum given to animal[a]	Challenge dose[b] (no. of organisms)	Outcome of challenge[a]
Rabbit		
R4P	10^6	G; dead by day 2
	10^7	G; dead by day 1
R4(23-32)	10^6	G; dead by day 1
	10^7	G; dead by day 1
R5P	10^6	G; dead by day 1
	10^7	G; dead by day 1
R5(41)	10^6	G; dead by day 1
	10^7	G; dead by day 1
Equine		
1863	10^6	SA; killed on day 6
	10^7	SA; killed on day 6
Nil	10^5	G; dead by day 1
	10^6	G; dead by day 1
	10^7	G; dead by day 1

[a] See footnotes to table VII/XI.

[b] See footnotes to table VII/I.

Fig. VII/1 - Microscopic appearances of acute gangrenous infection with Clostridium perfringens strain L2Ab (x 1250). Gram stain of material from infected thigh muscle of a guinea pig that died within 24 h after inoculation of 10^6 cells with adrenaline as initiating agent (control animal in table VII/XIII).

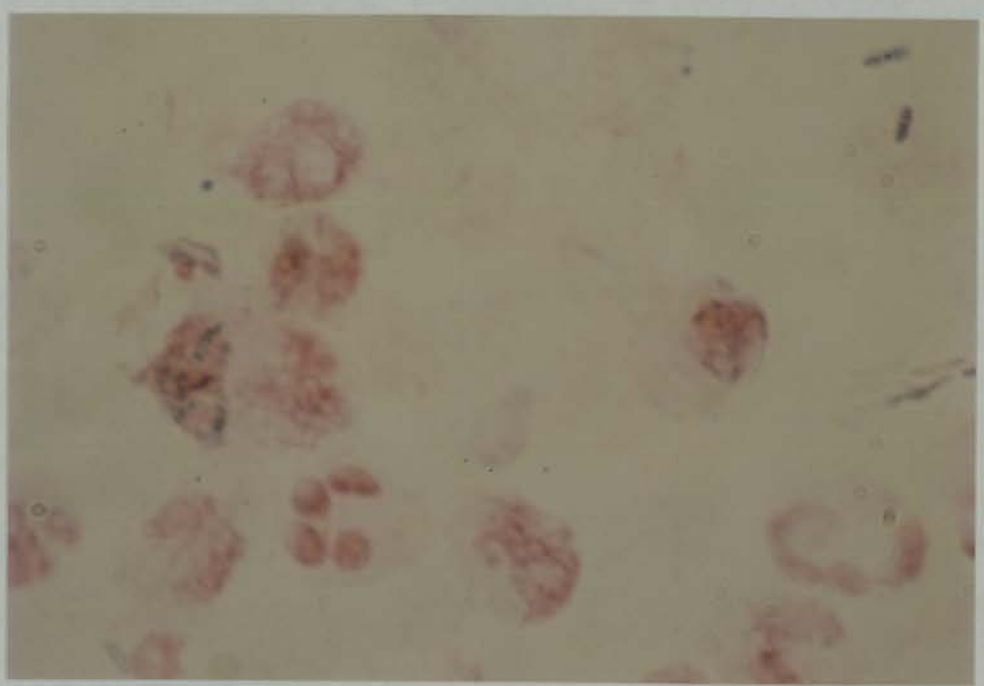


Fig. VII/2 - Microscopic appearances of localised infection with Clostridium perfringens strain L2Ab (x 1250). Gram stain of material from small abscess in thigh muscle of a guinea pig killed six days after inoculation with 10^7 cells with adrenaline as initiating agent but protected by equine serum 1863 (see table VII/XIII).

VIIa-c). In animals that developed acute gangrenous infection and died within 24-48 h, the films contained many Gram-positive bacilli with tissue debris but very few polymorphonuclear leucocytes (e.g. fig. VII/1); the same picture was seen whether $CaCl_2$ or adrenaline was used for initiation of infection. By contrast, in animals that survived challenge and contained the infection in an abscess localised at the site of inoculation, the films showed many pus cells with smaller numbers of extracellular bacilli, often degenerate, and evidence of intracellular bacterial debris (e.g. fig. VII/2). This picture was seen in animals given sublethal challenge doses of virulent strains and where antisera gave protection against an otherwise lethal challenge; it was also seen with large doses of less virulent strains where the injection of $CaCl_2$ provided a focus for the establishment of low-grade infection. It seems probable that the first few hours are critical in determining the outcome of challenge in these models of infection. Animals that survive have been able to mount an effective phagocytic response and eliminate or contain the challenge organisms; those that die have not. Antisera may tip the balance and protect the animal in the early stages so that effective defences can be mobilised and infection can be contained; our results do not suggest that anti-neuraminidase plays an important part in conferring this protection.

DISCUSSION

DEVELOPMENT OF HUMAN GLYCOPROTEIN SUBSTRATE AND NEURAMINIDASE ASSAY PROCEDURES

The results presented in Sections I and II describe the development of a sensitive assay procedure that was suitable for detection of neuraminidase activity in bacterial products or extracts of infected tissue, and that could be adapted to demonstrate inhibition of enzyme activity by antibody. Neuraminidases are unusual in the very wide range of substrates and assay procedures that can be used for detection of the enzyme (see reviews by Rafelson et al., 1966; Gottschalk & Bhargava, 1971; Drzeniek, 1972, 1973; Gottschalk & Drzeniek, 1972; Schauer, 1982). The early assays, using indirect systems such as destruction of virus receptors on red cells, were replaced by assays that measure directly the release of sialic acid from an appropriate sialic-acid-containing substrate. The assay system developed for the present work used a preparation of human α_1-acid glycoprotein that is cheap and can be made available in large amounts; it proved to be an excellent substrate in our assays.

Assay for NANA

Methods for assay of sialic acids have been reviewed by Tuppy & Gottschalk (1972), Ledeen & Yu (1976) and Schauer (1978). Most of the available procedures detect both free and bound molecules; assays for neuraminidase require a procedure that detects only the

free sialic acid released from an excess of substrate by action of the enzyme. The most frequently used assay system is the periodic acid/thiobarbituric acid reaction described by Aminoff (1959, 1961) and Warren (1959, 1963); various modifications have been suggested to increase the sensitivity and it has been adapted for automated assay systems and micro-techniques (Kendal & Madeley, 1969; Fidgen, 1973; Schauer, 1978). Other methods of estimating free sialic acid include the use of the enzyme N-acylneuraminate pyruvate-lyase (NAN-lyase); the amount of free NANA is reflected in the amount of pyruvate produced, which is determined by DPNH oxidation in the presence of lactate dehydrogenase (Brunetti et al., 1963; Ledeen & Yu, 1976). An alternative approach with sialyl-lactose as substrate is to assay for the release of lactose rather than of sialic acid (Holmquist, 1969). Synthetic chromogenic substrates, e.g. p-methoxyphenyl and 4-methylumbelliferyl compounds (Tuppy & Palese, 1969; Potier et al., 1979), and various radioactive-labelled substrates (Bernacki & Bosmann, 1973; Bhavanandan, Yeh & Carubelli, 1975; Schauer et al., 1976; Frisch & Neufeld, 1979) have also been developed, allowing detection of enzyme action by other techniques. These procedures may be more sensitive and more specific in particular circumstances, e.g. in detecting with confidence very low amounts of neuraminidase activity in mammalian tissue fractions. However, they also pose their own problems and many require expensive substrates that are not commercially available, or involve separation procedures that make them no less time-consuming than the original thiobarbituric acid assay.

The present studies used Aminoff's thiobarbituric acid assay for free NANA as described by Cassidy et al (1966; see Materials

and Methods). A number of factors are known that may interfere with this assay. The colour yield (E_{549}) is reduced in the presence of L-fucose, DNA, ferrous ions and a variety of other chemicals, and false positive results may occur with 2-keto-3-deoxyaldulonic acids, which give rise to the same chromophore (Warren, 1959; Hartree & Brown, 1970; Ledeen & Yu, 1976; Cabezas, 1978). False chromogens may give rise to other reddish products that give significant absorption at 549 nm; these include sucrose, 2-deoxyribose, malonaldehyde and unsaturated fatty acids (Warren, 1959; Horvat & Touster, 1968; Schauer, 1982). Warren (1959) recommended the use of a correction factor based on the absorption value at 532 nm to compensate for this but it is accurate only with low concentrations of interfering substances.

Factors such as the above are presumably responsible for the depression in assay value for NANA in the presence of various culture media and bacterial products that was observed in the present studies (Section IIa). Different batches of PPW5 broth vary in the extent to which they depress the NANA assay and the effect of the P9 culture filtrate shown in table II/I is the most marked that was observed; the effect is minimal with dialysed preparations or with purified Sigma neuraminidase. The mechanism of depression of the NANA assay in our studies was not investigated further but the possibility of this effect should be taken into account when neuraminidase assays are performed with unpurified enzyme preparations. In the present studies with crude culture materials and tissue extracts, care was taken to include control tests in order to determine the basal value (E_{549}) before enzyme action (Section IIc); high initial readings might have been

attributable either to free sialic acid or, more commonly, to the presence of other interfering substances in the test material. It was important to demonstrate clearly that an increase in assay reading during incubation of the tests was due to release of a product with peak absorption at 549 nm, and this was done in all cases where crude materials or products of previously uncharacterised bacterial species were examined.

A further source of error with the thiobarbituric acid assay occurs if there is NAN-lyase activity in the test material (Section IIb), since the NANA released by neuraminidase action might be destroyed during incubation of the reaction mixture (e.g. see fig. III/6). Nees et al. (1976) found that NAN-lyase activity was inhibited by pyruvate and recommended the addition of 10mM pyruvate in order to allow assay of neuraminidase when both enzymes might be present. Contaminating NAN-lyase may be one of the major indications for using a different type of assay procedure, e.g. with a chromogenic or radioactive substrate; however, the present studies included careful tests to assess this possibility and no insuperable problems were encountered.

Development of human glycoprotein substrate

No single neuraminidase assay procedure is ideal for all purposes. As outlined in the Introductory Review, neuraminidases from different sources vary quite markedly in substrate specificity, with their activity dependent on the particular N- and O-acyl substituents in the sialic acid, the specific α-glycosidic linkage to the neighbouring sugar, and the nature of the

glycoconjugate involved (protein or lipid). Most naturally occurring sialoglycoconjugates have some degree of heterogeneity, with a variety of linkages and sialic acids; in some cases these occur even within the same molecule. For detailed characterisation of enzymes from different sources it is desirable to use a battery of purified oligosaccharides, glycopeptides and gangliosides with defined N-acetyl and N-glycolyl sialic acids, and defined (2 → 3), (2 → 6) or (2 → 8) linkages (e.g. Corfield & Schauer, 1982b; Schauer, 1982). However, this is obviously impractical in screening assays for detection of neuraminidase in bacterial culture products when a broad-spectrum, general-purpose assay is required.

One of the simplest substrates is the oligosaccharide sialyllactose prepared from bovine colostrum; c. 80% of the sialic acid is (2 → 3) linked and 20% in the (2 → 6) isomer (Schneir & Rafelson, 1966). Most, but not all, neuraminidases have greatest activity against the (2 → 3) linkage; bacterial neuraminidases are generally of broad specificity though viral enzymes are markedly less active with the (2 → 6) linkage (see Introductory Review). Drzeniek (1973) recommended the use of purified N-acetylneuraminyl(2 → 3)lactose as standard substrate for neuraminidase assays, though many have continued to use the cruder mixture of both isomers. Glycoprotein substrates of animal origin in various degrees of purification have also been widely used, e.g. α_1-acid glycoprotein (orosomucoid), fetuin, submandibular gland glycoprotein or glycopeptides from edible-bird's-nest substance (Collocalia mucoid; see Kathan & Weeks, 1969). It may be argued that glycoprotein substrates provide a closer parallel to the

natural action of microbial neuraminidases than does NAN-lactose, a unique compound found only in colostrum (and the neonatal gut) of mammalian species. In some cases higher Km and Vmax values have been found with glycoproteins than with NAN-lactose as substrate (Drzeniek, 1972; Rosenberg & Schengrund, 1976b). For studies of enzymes with predominantly glycolipid activity, ganglioside preparations are more appropriate (Drzeniek, 1973; Schauer, 1982). Colominic acid has been used in screening for enzymes with good activity against (2 → 8) linkages (Uchida et al., 1974).

It is desirable to use a substrate that contains only N-acetyl neuraminic acid, since N-glycolyl and some O-acyl sialic acids give lower readings in thiobarbituric acid assays (Warren, 1959; Aminoff, 1961; Drzeniek, 1972; Schauer, 1982). For this reason substrates derived from human materials may be preferable to those from many other convenient animal sources, e.g. cattle, pigs and horses, which also contain a proportion of N-glycolyl derivatives. Walkowiak, Kedzierska & Starzinski (1968) claimed to have detected trace amounts of N-glycolyl neuraminic acid in human serum, but this has not been confirmed and it is generally agreed that N-glycolyl sialic acids do not occur in man (Ledeen & Yu, 1976; Corfield & Schauer, 1982a).

For the present studies a human α_1-acid glycoprotein substrate that could be easily prepared from a waste product of large-scale plasma fractionation was developed. Popenoe & Drew (1957) used α_1-acid glycoprotein prepared from the urine of nephrotic patients as substrate for C. perfringens neuraminidase, and Hughes & Jeanloz (1964) prepared it from human plasma for studies with pneumococcal neuraminidase. Cassidy, Jourdian &

Roseman (1965) purified and characterised the neuraminidase of C. perfringens; they recommended NAN-lactose as substrate for assays but also studied the release of NANA from α_1-acid glycoprotein. In early work on C. perfringens neuraminidase in this laboratory (Collee, 1962, 1965b), hen egg-white was used as substrate. This was later replaced by human plasma (Gadalla & Collee, 1968), and Collee & Barr (1968) then described a simple method of preparing an unrefined glycoprotein fraction from 200-400 ml of plasma from outdated human blood by trichloracetic acid precipitation. This human glycoprotein preparation gave more reliable results than were obtained with whole plasma, but the problems of scaling up certain stages, e.g. dialysis, would make it difficult to ensure a supply of closely standardised substrate during a prolonged investigation.

Preparation of the FVII glycoprotein substrate was undertaken in collaboration with Dr J.K. Smith, Protein Fractionation Centre, Scottish National Blood Transfusion Service (see Fraser & Smith, 1975, included in the Appendix). The fractionation procedures described in Section Ia were done by Dr Smith in the Protein Fractionation Centre; sialic acid analyses and evaluation as substrate for neuraminidase assays were done in this laboratory. Preliminary attempts to recover glycoproteins from Cohn fraction IV were unsuccessful but the one-stage FVII preparation from the supernate of fraction V proved much simpler. It was found to contain predominantly α_1-acid glycoprotein and also some albumin. A method for purification of α_1-acid glycoprotein from the same starting material, involving DEAE-cellulose adsorption and chromatography on CM-cellulose, has also been reported (Hao &

Wickerhauser, 1973). An alternative method of recovering pure α_1-acid glycoprotein from Cohn fraction VI involves the use of zinc salts, the residues of which might be undesirable in enzyme reaction mixtures (Bezkorovainy & Winzler, 1961; Schmid, 1975). The gel filtration and electrophoretic data indicate that the glycoprotein in our preparation was largely excluded from Sephadex G-200; native α_1-acid glycoprotein (MW 44,000) would not be. It is assumed that much of the glycoprotein in the FVII preparation is polymerised as a result of the pasteurisation procedure (Spragg et al., 1969); this partial denaturation might facilitate hydrolysis by neuraminidase. Although the probability of transmission of serum hepatitis by this fraction is relatively small (Berg et al., 1972) there is a risk that any product from pooled human serum may contain hepatitis or other viruses; all such products should have been pasteurised (60°C for 10 h) at some stage before use in normal laboratory conditions.

Human α_1-acid glycoprotein contains c. 40% of carbohydrate; NANA constitutes 11-12% of the total weight of the protein. Rather more than 50% of the NANA is in the (2→3) linkage and the rest predominantly in the (2→6) linkage; variation in linkage of NANA is responsible for electrophoretic microheterogeneity (Jeanloz, 1972; Schmid, 1975). Although the presence of (2→6) linkages might be a disadvantage in studies with viral neuraminidase, it might be an advantage in screening for bacterial enzymes with their broader substrate range. Popenoe & Drew (1957) and Cassidy et al. (1965) found that all of the NANA could be released from α_1-acid glycoprotein by C. perfringens neuraminidase. The results presented in Section Ib show that C. perfringens neuraminidase can

also release all the NANA from our FVII preparation. Mild acid hydrolysis is recommended for complete removal of sialic acid from sialoglycoconjugates but many such procedures also give significant degradation of the released NANA, and allowance has to be made for this in calculating the results (Schauer, 1982). Our procedure, using 0.02M H_2SO_4 at 80°C gave complete hydrolysis from the FVII substrate within 1-2 h without significant destruction of the released NANA (fig. I/3); with stronger acid or higher temperature there was no increase in the total released but there was significant NANA degradation (e.g. see fig. I/4). Comparison of the results in figs I/3 and I/5 confirms that our stock preparation of C. perfringens neuraminidase could release the same amount of NANA from the FVII substrate as was released by acid hydrolysis.

The enzyme-substrate kinetic studies described in Section Ic showed that the FVII preparation is a good substrate for the C. perfringens enzyme. Calculations based on the observed activity of the commercially available purified enzyme (Sigma) with our substrate suggest that the initial velocity with FVII substrate is comparable to that with bovine NAN-lactose (Section IIf). Cassidy et al. (1965) found the initial rate of release of NANA from α_1-acid glycoprotein to be slightly faster than from NAN-lactose; Schauer et al. (1976) also found a radioactive-labelled α_1-acid glycoprotein preparation to be a good substrate for C. perfringens neuraminidase. Fig. I/9 shows that there was linear release of NANA from an excess of substrate when concentrations of enzyme in the range of c. 2-20 milliunits/ml were tested in assays incubated at 37°C for periods between 10 and 40 min. The thiobarbituric acid assay becomes less accurate at readings (E_{549}) above c. 0.5 (see

fig. II/3) but with a 15-min assay it was possible to measure up to c. 50 milliunits/ml quite accurately; more concentrated samples should be diluted before assay so that the results fall in the reliable range. Even very small amounts of neuraminidase activity (c. 0.1 milliunit/ml) can be detected with confidence by prolonging the period of incubation for up to 24 h (figs I/10 and 11); this is of great value when it is important to demonstrate the absence of the enzyme in defining neuraminidase-negative strains of bacteria.

A number of batches of FVII substrate were prepared, differing only in minor details of scale. With substrate batch FVII(5), Vmax occurred at concentrations of c. 3 mg FVII/ml (Section Ic), equivalent to c. 140 µg releasable NANA/ml. Further batches of substrate were used at concentrations containing 140-170 µg NANA/ml (see table I/III). Comparison of different batches of substrate (Section Id) showed them to be very similar; the concentration of NANA in batch FVII(7) was rather low, and assays with this batch of substrate might also be a little lower. Nevertheless, with meticulous assay technique and appropriate control tests used to correct the assay values, it was possible to produce results that gave a linear correlation with enzyme activity up to values (E_{549}) of c. 0.4-0.5 (fig. I/12).

The pasteurised FVII preparation is not an ideal substrate in that it does not consist of a pure well characterised substance, but it possesses certain compensating advantages. It is produced from a waste fraction and can therefore be made available comparatively cheaply. (In the United Kingdom, with its important tradition of volunteer blood donors, blood products cannot be sold

commercially; special permission was obtained to allow a nominal charge for the FVII substrate to defray the cost of recovering this product from otherwise waste material.) It is free from the risk of hepatitis virus transmission, is easy to prepare in standard form in very large quantities and is very stable as a freeze-dried powder and even in solution at 4°C. It has a low ionic content, the only residual buffer being a small amount of acetate, and the pH and ionic strength of the reaction mixture can therefore be easily adjusted. The content of readily hydrolysable NANA is high enough to avoid problems of viscosity or turbidity, and the level of free chromogen before hydrolysis is conveniently low. It should be borne in mind that the FVII preparation may contain a mixture of glycoproteins and of glycosidic linkages that may be hydrolysed at different rates by the same or by different enzymes. Nevertheless, it has proved to be a good substrate for the neuraminidase of clostridia, Bacteroides species and Vibrio cholerae in the studies reported here, and also for myxovirus and Corynebacterium diphtheriae neuraminidase in other hands (Fidgen, 1975).

Assay procedure for C. perfringens neuraminidase

The development of standardised procedures for assay of C. perfringens neuraminidase with the FVII substrate is described in Section II. Studies with the commercially available purified enzyme preparation (Sigma) suggested that the FVII substrate gives assay values comparable to those obtained with NAN-lactose, and considerably higher than with bovine submaxillary mucin (Section IIf). However, the purified Sigma enzyme is expensive, and

unstable when held in solution; further studies to determine optimal conditions for the neuraminidase assay were performed with unpurified enzyme preparations derived from the filtered supernate of a PPW5 broth culture of C. perfringens type A strain L2Ab (see Materials and Methods). Samples of the original bulk preparation (P9) were dialysed in order to remove substances that interfere with the thiobarbituric acid assay (Section IIa). These preparations had good neuraminidase activity (c. 20 milliunits/ml; table II/V) and did not contain detectable phospholipase-C; they proved stable for long periods at -20°C (Section IIf) and were used as stock enzyme preparations throughout these studies.

The control tests used for correcting neuraminidase assay results are discussed in Section IIc. Separate enzyme and substrate control tests incubated at 37°C for the same period of time as the reaction mixture give the most accurate results for correction of standard assays. This is impractical for progress experiments because of the inaccuracies in timing of addition of assay reagents that would result from inclusion of too many tubes in a single batch of assays; conditions under which time-zero (T_0) control tests give accurate results were carefully defined. In general, test and control assays were performed in duplicate and average values were used in calculation of results; this minimised the chances of error arising from an occasional anomalous test or control assay.

The results given in table II/IV confirm that C. perfringens neuraminidase, in contrast to the Vibrio cholerae enzyme, does not require added Ca^{2+} (Cassidy et al., 1965; Drzeniek, 1972); even the addition of 5mM EDTA (Boschman & Jacobs, 1965) reduced the

assay value only by c. 20%. Ca^{2+} was not added to our assays as a routine; however, occasional tests were repeated with added Ca^{2+} to confirm that apparently negative results might not conceal a calcium-sensitive enzyme.

Varying the pH of our assay in tris-maleate buffer produced a plot of activity that showed a simple curve with a clear-cut optimum at c. pH 5.7; when sodium acetate buffer was used the curve was more complex with the main peak at c. pH 4.7 and a subsidiary peak at c. pH 5.6 (fig. II/7). The pH optimum for the activity of a particular neuraminidase may vary considerably when tested with different substrates (Rafelson et al., 1963; Uchida et al., 1979) or in the presence of different buffer ions (Cassidy et al., 1965; Drzeniek, 1972). Burton (1963) found a sharp optimum at pH 5.0-5.1 for C. perfringens neuraminidase tested with a ganglioside substrate in acetate buffer. Nees et al. (1975), with Collocalia mucoid and acetate buffer, reported optimum activity between pH 4.3 and 5.2. Popenoe & Drew (1957) found the optimum to be pH 5.0-5.5 in acetate buffer with α_1-acid glycoprotein. Cassidy et al. (1965) reported the optimum for C. perfringens neuraminidase acting on bovine NAN-lactose to be at c. pH 5.6 in tris-maleate or in citrate-phosphate buffers, but pH 4.0-4.8 in acetate buffer. Our finding of a subsidiary peak around pH 5.6 in acetate buffer with the FVII substrate may reflect a heterogeneity of NANA linkages in the preparation, perhaps due to the presence of a mixture of glycoproteins or to alterations in glycoproteins produced by pasteurisation; it might also be due to different pH optima for the hydrolysis of (2→3) and (2→6) linkages, as was found for myxovirus neuraminidase by Schneir & Rafelson (1966).

When the assay is done with FVII substrate and sodium acetate buffer at pH 5.1 the results fall on the high plateau between the two peaks of activity and would not be markedly influenced by slight divergence from this value in reaction mixtures. A very much lower pH (below pH 4.2) would be required if it was desired to prevent possible interference with the assay for neuraminidase by contaminating NAN-lyase (see fig. II/5).

Neuraminidase activity is commonly expressed in terms of units; 1 unit liberates 1 μmole of NANA/min from the substrate at 37°C in a suitable buffer at the optimum pH (Drzeniek, 1972). Maximum activity of the stock enzyme preparation P9D3 occurred at temperatures of 45-55°C (fig. II/6) but our standard assays were performed at 37°C. It seems desirable that enzyme activities should be stated in standard units measured with NAN(2 → 3)lactose as substrate and we have refrained from expressing our results routinely in terms of units measured with our substrate; the presence of interfering substances or NAN-lyase activity further complicate the accurate calculation of neuraminidase activity in unpurified bacterial culture products or tissue extracts. However, reference assays of 10 μg pure NANA were used for calculation of the observed activity of our stock enzyme preparations so that they could be related to that of the reference Sigma preparation (tables II/V and VI).

For reliable results, only tests performed in a single batch should be regarded as strictly comparable; careful attention to detail was necessary in order to produce accurate and consistent results such as are presented in Sections I and II. The results presented in fig. II/8 show the reproducibility that could be

achieved with carefully standardised neuraminidase assays on different occasions over a period of some weeks; however in series of tests performed over longer periods of time results were found to be much more variable. Reference assays with standard tubes containing 10 µg pure NANA were included with all batches of neuraminidase assays (Section IIa). Variations in the values obtained with these reference assays for NANA, and with our stock neuraminidase preparations, might be attributable to different batches of assay reagents, minor inaccuracies in timing, temperature or volumes of reagent added at different stages of the assay for NANA, or differences in pH or temperature of incubation of the enzyme reaction mixture. For most purposes such variation in values for reference assays was not important, since inaccuracies due to the presence of interfering substances or NAN-lyase in the test material, or variations in production of the enzyme in different culture media by the test organism, were likely to be greater. The standardised assay procedures adopted for use with the FVII substrate proved very satisfactory when applied, with a semi-quantitative scale (see Materials and Methods), to studies of neuraminidase production by a wide range of clostridial and bacteroides strains in the next part of this investigation.

PRODUCTION OF NEURAMINIDASE BY ANAEROBIC BACTERIA

Production of neuraminidase by C. perfringens

The results of experiments investigating production of the enzyme by strain L2Ab, a classical gas-gangrene-producing strain of C. perfringens type A, are reported in Section IIIa. It was confirmed that C. perfringens neuraminidase is an exoenzyme produced in large amounts in the supernate of cultures in appropriate media (Cassidy et al., 1965; Nees & Schauer, 1974b). Exoenzymes and exotoxins are classically produced by intact cells during log-phase growth and this was demonstrated in the growth-curve experiment shown in figs III/1 and 2; the level reached a maximum during early stationary phase and did not increase further after 24 h although the numbers of viable cells began to decline. The enzyme is predominantly in the culture supernate; other experiments (e.g. table III/X) showed that only a little neuraminidase remains cell-associated after thorough washing of the cells. A few clostridial products, notably the enterotoxin associated with food-poisoning strains of C. perfringens type A, are not produced during log-phase growth but are directly related to sporulation (Duncan, Strong & Sebald, 1972). Most strains of C. perfringens produce few spores during culture in ordinary laboratory media; in this experiment they formed only c. 0.1% of the total L2Ab cells present even after incubation for 72 h (fig. III/1). Although production of spores started in late log phase it is clear that considerable amounts of the enzyme had already

appeared before spores became detectable and there was no indication that neuraminidase production is related to sporulation.

The assay procedures developed for detection of NAN-lyase in these studies were intended primarily to assess inaccuracies in the neuraminidase assay attributable to contaminating NAN-lyase rather than to give a sensitive assay for the enzyme; however, the results presented in Section IIb confirm that NAN-lyase remains predominantly cell-associated in C. perfringens cultures (Comb & Roseman, 1960; Nees & Schauer, 1974a). There was no evidence of NAN-lyase activity in the stock P9 enzyme preparations (table II/I) and none was detected in the experiment shown in fig. III/1 even at 72 h when the viable count was falling and cell lysis might be expected. However, Popenoe & Drew (1957) found a little NAN-lyase in culture filtrates of C. perfringens, and some NANA-destroying activity was occasionally detected in culture supernates of strain L2Ab and other clostridial strains in the present studies (e.g. see table III/XI); this possibility was carefully checked when low-positive or negative results were obtained in assays of bacterial products for neuraminidase.

Neuraminidase was generally well produced by C. perfringens strains in several broth media but considerable variations in yield were found even under apparently identical culture conditions. The results given in table III/I showed that the assay values in replicate cultures in the same batch of medium incubated in the same anaerobic jar might vary by c. 10% from the mean; in serial cultures much greater variation was seen (e.g. fig. III/4). It proved difficult to identify or control factors responsible for such variation and this made it difficult to define conditions that

would consistently give maximum yields of the enzyme. Cultures were incubated for 48 h as a routine; the results of the growth-curve experiment (fig. III/2), with a larger volume of culture than in the standard tests, suggest that maximum enzyme activity is achieved well before 48 h and that it remains very stable in the culture supernate thereafter. Fig. III/3 shows that the temperature of incubation of the culture markedly influences neuraminidase production; some inaccuracy in temperature control may be hard to avoid when using anaerobic jars in conventional incubators in a busy laboratory and this was probably responsible for some of the variation encountered in the present studies.

Neuraminidase has been shown to be an inducible enzyme in several bacterial species (see Introductory Review). Nees & Schauer (1974a), using a synthetic basal medium in which their test strain of C. perfringens produced very low levels of neuraminidase and NAN-lyase, showed co-ordinated induction of both enzymes when free NANA or Collocalia sialopeptides were added in a chemostat system. The glycopeptide-bound NANA gave more rapid induction and higher enzyme levels than free NANA; N-acetyl mannosamine had a very low inductive effect with this organism, in contrast to the situation with Vibrio cholerae (Ada & French, 1959), pneumococci (Kelly et al., 1966) and Pasteurella multocida (Drzeniek et al., 1972). Schauer (1982) postulated that free NANA inside the cell is the inducer for the enzymes in C. perfringens; the delay in induction by free NANA added to the external medium reflects the time taken to induce a hypothetical permease for efficient uptake of NANA. The rapid inductive effect of sialopeptides suggests that these can be taken up directly by the cell, and do not depend on

extracellular hydrolysis and subsequent uptake of the released NANA. He further suggested that protease production by clostridia would act in co-operation with this system, releasing sialopeptides from glycoproteins in the environment and thus triggering induction of the full system for NANA release, uptake and breakdown by the cells; it should be appreciated that C. perfringens type A is usually regarded as a non-proteolytic species, although it does have collagenase and gelatinase activity (see Willis, 1977, chap. 4). In the present studies it was not possible to identify inducing substances that would consistently produce higher yields of neuraminidase in the supernate of 48-h broth cultures (Section IIIa). Although on occasion the addition of free NANA, FVII glycoprotein or proteose peptone appeared to give increased final enzyme yields, this did not occur consistently and varied yields were obtained on different occasions with any particular medium.

Fig. III/4 shows that strain L2Ab produced more neuraminidase during serial cultures in PPW5 medium than in NB medium; there was a clear-cut step up or down in enzyme production after each change of medium. Neuraminidase production by classical C. perfringens strains was found to be generally good in PPW5 and THB media (table III/II) and these were used for further studies with clostridial species. Although it appeared that neuraminidase production might be enhanced by serial culture in some experiments (e.g. figs III/4 and 5), this was not found to be generally true (Section IIIa); however, the supernate of the second serial 48-h culture in PPW5 or THB medium was assayed as a routine in testing for production of the enzyme by clostridial species.

A number of other C. perfringens strains were also examined for neuraminidase production (Section IIIc). Collee (1965a) reported that the enzyme was produced by strains of types B, C, D and E as well as by type-A strains and this was confirmed in the present studies (table III/XIII). Strains of C. perfringens type A from various sources were characterised in order to select a range of organisms with different properties for later studies of virulence in experimental guinea pig infections. Strains NCTC11144, CW6 and CW7, three non-phospholipase-producing C. perfringens strains, all produced good levels of neuraminidase in vitro (table III/XIII). Rood & Wilkinson (1975) described mutant strains of C. perfringens that were neuraminidase-negative or were very weak producers of neuraminidase. A number of their strains were obtained and recharacterised in this laboratory (table III/XIV). Unfortunately, they all produced high yields of enzyme with our test procedures, perhaps because of genetic instability; they were not further investigated in the present studies. However, a number of naturally-occurring neuraminidase-negative strains were identified during studies with food-poisoning strains of C. perfringens (discussed below) and these were used in later studies of the correlation of virulence and neuraminidase production.

Production of neuraminidase by food-poisoning strains of C. perfringens type A

When the present investigation was undertaken there was confusion in the literature regarding the production of neura-

minidase by food-poisoning strains; this partly reflected the changing definition of such strains. C. perfringens type A occurs regularly as a commensal in the gut of man and animals, and this for long delayed recognition of its role as a pathogen in the human gut (for reviews of C. perfringens food poisoning see Hauschild, 1974; Willis, 1977, chap. 7; Hobbs, 1979; McDonel, 1980: see also Fraser & Collee, 1979, included in the Appendix). The work of Hobbs et al. (1953) in Britain finally established the organism as a cause of food poisoning; the "typical food-poisoning strains" that were implicated could be distinguished because they grew as non-haemolytic colonies on horse-blood agar and produced unusually heat-resistant spores. Such organisms have been classified as type-A_2, to distinguish them from classical strains of type-A_1 (Sterne & Warrack, 1964). Although type-A_2 strains are the more likely to survive cooking and cause food poisoning, later studies in Britain and America established that food poisoning can also be produced by classical type-A_1 strains that produce relatively heat-sensitive spores (Hall et al., 1963; Hauschild & Thatcher, 1967; Sutton & Hobbs, 1968). It is now clear that the potential to cause food poisoning depends upon the ability to produce enterotoxin during sporulation in the gut, and that both haemolytic and non-haemolytic strains may do this (Hauschild, Niilo & Dorward, 1967, 1971; Duncan & Strong, 1969a and b).

The sensitive assay procedures developed with C. perfringens strain L2Ab were used for investigation of neuraminidase production by reference British food-poisoning strains of C. perfringens sero-types 1-24 (heat-resistant) and i-xviii (heat-sensitive); this work was reported by Fraser & Collee (1975, included in the

Appendix). Various attempts have been made to develop serotyping methods for C. perfringens strains (see Willis, 1969, chap. 2; Chakrabarty & Narayan, 1979). Two sets of antisera were developed in the Central Public Health Laboratory, Colindale, for epidemiological studies on type-A_2 or type-A_1 food-poisoning strains (Hobbs et al., 1953, 1973) but there was some overlap between the two groups and more recent efforts have been aimed at producing a unified set of antisera for all C. perfringens type A strains (Hughes, Turnbull & Stringer, 1976; Stringer, Turnbull & Gilbert, 1980). Table Disc/I shows the revised numbers allocated to the strains previously given Roman serotype numbers; for clarity the original designations have been retained in the present report.

Collee (1965b) found that four British type-A_2 food-poisoning strains were neuraminidase-negative but, because culture supernates of two strains produced a myxovirus-receptor-inactivating effect on red cells (Collee, 1965a), he concluded that "more extensive investigation is required before it can be claimed that neuraminidase is never produced by typical food-poisoning strains". Moss, Schekter & Cherry (1967) confirmed that 12 British food-poisoning strains (the reference strains of Hobbs' types 1-3 and 5-13) were neuraminidase-negative; however they went on to demonstrate that one of five American food-poisoning isolates that were otherwise indistinguishable from the British type-A_2 strains did produce the enzyme. American food-poisoning isolates generally include many that are haemolytic or heat-sensitive or both; the majority of the American food-poisoning strains examined by Moss et al. (1967) were neuraminidase-positive.

TABLE Disc/I

Revised serotype designations for heat-sensitive

Clostridium perfringens type A

Former type number	New type number[a]
i, xii	23
ii	24
iii, v	25
vi	26
vii	38
viii	27
ix	28
x	29
xi	39
xiii	17
xiv	4
xv	9
xvi	40
xvii	30
xviii	31

[a] From Hughes et al. (1976); former strain iv was lost and is no longer included.

Table III/V contains the results of tests with the British reference food-poisoning strains cultured in PPW5 broth; the relative amounts of enzyme produced by neuraminidase-positive strains are shown in table III/IV. Twenty of 24 heat-resistant strains were neuraminidase-negative, whereas 12 of 17 so-called heat-sensitive strains produced the enzyme; thus, in British as in American strains, the ability to cause food poisoning is not strictly correlated with lack of neuraminidase production, although the majority of non-haemolytic, heat-resistant isolates are neuraminidase-negative.

The four heat-resistant strains that were found to be clearly neuraminidase-positive include the reference strain of Hobbs' type 13, which was reported not to produce the enzyme by Moss et al. (1967). Three other type-13 strains also produced neuraminidase (table III/VI), including strains 029 and 153 which were amongst the strains reported as negative by Collee (1965b). It is possible that these strains might have altered as a result of prolonged storage and subculture, although their characteristics were carefully rechecked to confirm that they were still non-haemolytic and produced heat-resistant spores. PPW5 broth is generally much better for the production of neuraminidase than the media used by Collee in 1965 and it is unlikely that the less sensitive assay procedure then in use, with egg-white as substrate, would have detected the small amounts of neuraminidase produced in some cultures of these strains even in PPW5 broth, e.g. in the experiment shown in fig. III/5. Although the results in this experiment suggested that sequential subculture in PPW5 broth might result in a gradually increased yield of the enzyme, this could not

be confirmed in further studies (e.g. see table III/VII); there was considerable variation in the amount of enzyme produced by strains 029 and 153 on different occasions but all cultures examined could be clearly shown to be neuraminidase-positive. It is probable that the difference between the present findings and those of Collee (1965b) are attributable to developments in culture and assay procedures. However, it appears that there may be variation in the ability to produce neuraminidase even among strains of a single C. perfringens serotype as tests with strain 4621 (also Hobbs' serotype 13) showed no evidence of neuraminidase production even after incubation of assays for 24 h (table III/VI). A further example emerges from the amalgamation of the former sets of serotype strains shown in table Disc/I. Strains i and xii, both neuraminidase-positive, have now been reallocated to serotype 23; however, the original type-23 reference strain was neuraminidase-negative in the present survey (table III/V).

The strains of C. perfringens listed in table III/IV are clearly neuraminidase-positive; it is more difficult to be certain that an organism is truly neuraminidase-negative and can never produce the enzyme. Because of the variations in enzyme production seen in cultures of neuraminidase-positive strains, four apparently negative strains (Hobbs' serotypes 1-4) were also examined in five serial cultures in PPW5 broth undertaken as part of the experiment shown in fig. III/5; no enzyme could be demonstrated even with assays incubated for 24 h (table III/VIII). The absence of detectable enzyme was confirmed in further serial cultures of the Hobbs' type-2 strain grown at 39°C under conditions that gave very

high enzyme levels with strain L2Ab (table III/X). Even with the culture supernate concentrated 5-fold no extracellular enzyme was detected, and no neuraminidase was released from the cells by ultrasonic disintegration. Further studies showed no NAN-lyase activity in the culture supernates (table III/XI); this demonstrates the validity of the 24-h assays and confirms that the Hobbs` type-2 strain produces no extracellular neuraminidase (i.e. <0.001% of the neuraminidase produced by strain L2Ab). When the L2Ab cells were disrupted, the NAN-lyase that was released produced some interference with the neuraminidase assay (table III/XII; fig. III/6). A similar amount of NAN-lyase was released from the neuraminidase-negative Hobbs` type-2 strain, but further experiments showed that this would not prevent the demonstration of quite small amounts of neuraminidase - as little as 1% of that associated with strain L2Ab cells (fig. III/7).

These studies demonstrated that British food-poisoning strains of C. perfringens type A include some that are neuraminidase-positive and others that still cannot be shown to produce the enzyme in vitro. Satterlee & Walker (1969) suggested that C. perfringens neuraminidase might be the factor producing increased intestinal passage time in mice fed on a diet to which the organism had been added. However, since many food-poisoning strains do not produce the enzyme, and since the role of the enterotoxin has since been clearly attested, it is unlikely that neuraminidase is involved in the mechanism of pathogenicity of C. perfringens type A in the gut.

Production of neuraminidase by other species of clostridia

The procedures developed for definition of neuraminidase-negative strains of C. perfringens were used in a study of a range of well characterised strains of other clostridial species (see Section IV, and Fraser, 1978, included in the Appendix). Neuraminidase-positive strains were shown to produce the enzyme in culture supernates in both PPW5 and THB media although there were often considerable differences in the relative amounts produced and these did not correlate clearly with differences in the relative amounts of growth in the two media. Neuraminidase-negative strains grew adequately in the test media but could not be shown to produce the enzyme in either, even when assays were incubated for 24 h; none of the culture products of these strains contained sufficient NAN-lyase activity to interfere significantly with the assays for neuraminidase. Assays were performed in acetate buffer at pH 5.1 by the standard procedure used for the C. perfringens enzyme and this proved satisfactory for detection of neuraminidase produced by other clostridial species. The enzyme produced by various clostridia was shown not to be calcium-dependent (table IV/III); Ca^{2+} was not added to the assays as a routine but culture products of a few neuraminidase-negative clostridia were retested with added Ca^{2+} as a precaution. Clostridial neuraminidase is essentially extracellular (tables IV/I and II) but several neuraminidase-negative strains were further tested to confirm that no cell-associated enzyme could be detected after disruption of the cells.

The results of these studies are summarised in table Disc/II. The strains and species of Clostridium tested are described in

TABLE Disc/II

Production of neuraminidase by clostridial species[a]

Neuraminidase-positive species	Neuraminidase-negative species
C. perfringens types A-E[b]	C. novyi types A-D (10)
C. septicum (5)	C. tetani (6)
C. chauvoei (2)	C. botulinum (4)
C. tertium (2)	C. difficile (6)
C. sordelli (12)	C. sporogenes (4)
C. absonum (3)	C. histolyticum (4)
	C. bifermentans (5)
	C. paraperfringens (4)
	C. butyricum (1)
	C. sphenoides (1)
	C. fallax (1)
	C. cochlearium (1)
	C. subterminale (1)

[a] Extracted from tables IV/I, II, V, VI and VII; number of strains tested indicated in brackets.

[b] Both neuraminidase-positive and -negative strains of C. perfringens type A occur (see table III/V).

Materials and Methods, where the procedures used for their identification are also detailed. The clostridial species that produced neuraminidase in the present study were C. septicum, C. chauvoei, C. tertium, C. sordelli and C. absonum; all strains of each species tested were neuraminidase-positive. Although these results were clear-cut and consistent it should be appreciated that relatively small numbers of strains of each species were investigated; it remains possible that a more exhaustive search might reveal neuraminidase-negative variants such as occur among food-poisoning strains of C. perfringens type A.

C. septicum and C. chauvoei are usually regarded as separate species although the differences between them are very small; Willis (1969, chap. 4) discussed the arguments for regarding them as two types of a single species. Warren & Spearing (1963) noted the presence of neuraminidase activity in a lyophilised preparation of C. septicum toxin. Gadalla & Collee (1968) found that the enzyme was produced by all of 15 strains studied, and Muller (1970b) demonstrated neuraminidase production by a strain of C. septicum isolated from a patient with a wound infection. Muller & Schallehn (1972) detected the enzyme in two strains of C. chauvoei by demonstrating characteristic electrophoretic changes in serum proteins included in the culture medium. The present study confirmed that strains of C. septicum generally produce large amounts of neuraminidase, whereas the two strains of C. chauvoei produced rather smaller amounts under the same test conditions (table IV/I).

Howe et al. (1957) reported that C. tertium produced an enzyme that destroyed myxovirus receptor substances. Muller &

Werner (1974) demonstrated neuraminidase production by a strain of C. tertium and the present study confirmed that small amounts of the enzyme were produced by two other strains (table IV/I).

C. sordelli and C. bifermentans are very closely related and there has been considerable debate as to whether or not they should be regarded as separate species (see Willis, 1969, chap. 5). Pathogenic strains of C. sordelli produce the β-toxin whereas C. bifermentans is non-toxigenic, but this is not an adequate criterion for classification as non-toxigenic strains of C. sordelli also occur; in the present studies only one of the five C. sordelli strains tested was found to produce the toxin (Section VIId). Nakamura et al. (1975) used numerical taxonomy and DNA homology in a reinvestigation of this problem. They scored for 162 features (not including neuraminidase activity) and concluded that the two species could be reliably differentiated by only a few biochemical tests - fermentation of mannose and sorbitol, urease activity, arginine deamination and growth inhibition by mannose. The identity of our strains was carefully rechecked in the present studies; minor inconsistencies were obtained with the fermentation tests for two C. bifermentans strains (see table IV/IV). Warren & Spearing (1963) noted neuraminidase activity in a preparation of C. sordelli toxin; White & Mellanby (1969) separated the neuraminidase from the toxic activity in the culture supernate of a strain of C. sordelli. The present finding that all of 12 strains of C. sordelli produced neuraminidase but that none of five strains of C. bifermentans did so (Section IVc), suggests that this may be another character that distinguishes the two species. C. difficile produces a cytotoxin that is antigenically related to the

C. sordelli toxin and neutralised by C. sordelli antitoxin (Bartlett et al., 1978; Bartlett, 1979; Burdon, 1981), and the two species have serologically related surface antigens (Poxton & Byrne, 1981); however, none of the C. difficile strains examined was found to produce neuraminidase.

C. absonum and C. paraperfringens are non-pathogenic species that are related to C. perfringens but can be distinguished by differences in their phospholipase reactions (Nakamura et al., 1973; Hayase et al., 1974). When grown on EYA medium, C. absonum strains produce a broad zone of opalescence that is only partially inhibited by C. perfringens antitoxin; C. paraperfringens strains produce a narrow zone of opalescence that is completely inhibited by the antiserum (see Materials and Methods). Nakamura et al. (1973) found that these species differed from each other and from C. perfringens in only a few other biochemical tests. It now appears that neuraminidase production may be a further distinguishing feature; C. absonum strains resembled C. perfringens in producing the enzyme whereas C. paraperfringens strains were neuraminidase-negative (tables IV/I and VII). Solovev et al. (1972) have suggested that tests for neuraminidase production may be of interest in studies of Vibrio taxonomy and it now appears that they may also be of value in certain aspects of clostridial taxonomy (see also Collee et al., 1982, included in the Appendix).

The other species of Clostridium found to be neuraminidase-negative in these studies are also shown in table Disc/II. Werner & Muller (1974) described a neuraminidase-positive strain of Clostridium pseudotetanicum; however, this is presumably different

from the C. tetanomorphum strain NCTC2909 included in table IV/VII, which is now reclassified as C. cochlearium (see Materials and Methods). The possible relationship of neuraminidase production to pathogenicity in clostridia is discussed later. However, it is clear that several important pathogenic species do not produce neuraminidase, e.g. the strains of C. novyi, C. tetani, C. botulinum and C. difficile were all neuraminidase-negative.

Production of neuraminidase by Bacteroidaceae

The importance of the Bacteroides-Fusobacterium group of Gram-negative anaerobic bacilli as significant pathogens in a variety of infections has become increasingly apparent with recent improvements in methods for their isolation and identification (Finegold, 1977). Little is known of their mechanisms of pathogenicity, and the biochemical attributes of the different species are still being defined. The studies of neuraminidase production by clostridia were extended to a range of well characterised strains representing the major species of Gram-negative non-sporing anaerobic bacilli (see Section V, and Fraser & Brown, 1981, included in the Appendix); the current classification of the test strains is detailed in Materials and Methods.

Section Va describes the results of experiments undertaken to characterise production of the enzyme by Bacteroides fragilis (B. fragilis ss. fragilis) strain NCTC9344. The enzyme was found to be predominantly cell-associated, with very little activity detectable in the supernates of 48-h cultures (table V/I); cell extracts contained strong activity and were tested in all further

studies with bacteroides strains. Hammann, Von Nicolai & Werner (1981) reviewed their group's studies with neuraminidase from several bacteroides strains; they found generally greater activity against NAN-lactose than glycoprotein substrates and reported pH optima of 5.6-6.5 with NAN-lactose in barbital-acetate buffers (Von Nicolai, Werner & Zilliken, 1977, 1978). Fig. V/1 shows that the optimum for B. fragilis neuraminidase in our assays with the FVII substrate and acetate buffer was c. pH 4.2; the standard assay procedures at pH 5.1 gave good results and were used throughout these studies. The enzyme was shown not to be calcium-dependent (table V/II) and Ca^{2+} was not added to the assays. Berg et al. (1983) have recently reported the properties of purified B. fragilis neuraminidase with NAN-lactose as substrate; they were able to demonstrate some stimulation of enzyme activity (by 20%) when $CaCl^{2+}$ was added and the pH optimum was found to be at pH 6.5. The discrepancies between their findings and the results in the present study may be attributable to differences in the substrate and buffer system employed.

Many of the Bacteroidaceae are nutritionally fastidious and do not grow well in fluid media even with the addition of growth supplements and meticulous anaerobic technique. The three media used in the present study were chosen for their ability to give good enzyme production by neuraminidase-positive species and also good growth of a wide range of Gram-negative anaerobes (Sections Vb and c). DB medium allowed adequate growth of all species tested but did not always give the best production of neuraminidase. Some of the B. fragilis group of organisms produced more enzyme in PPW5 broth; however, this medium did not support adequate growth of

several other species, even when incubation of the cultures was extended to 96 h. A few of these strains produced more enzyme in BM broth than in DB medium, but there were also clear exceptions. Growth was assessed by observation of turbidity in test cultures; a number of species, e.g. B. corrodens and Fusobacterium strains, do not give strong turbidity even when many cells are present, and this was allowed for in the scoring system for recording growth (see Materials and Methods). Neuraminidase-positive strains generally produced the enzyme in each of the two media in which they were grown, but a few proved variable or negative in one or the other (see tables V/III and IV). The purity of all cultures was carefully checked in subcultures from the test culture. Since no single medium consistently gave optimum production by neuraminidase-positive strains, other strains were tested in at least two media that supported adequate growth. All cell extracts were tested in assays incubated for 24 h before being classed as neuraminidase-negative.

Muller & Werner (1970b), using a paper chromatographic method, reported the presence of NAN-lyase in several species of Bacteroides, including B. fragilis, and Muller (1973b) also found activity with a strain of Fusobacterium polymorphum (F. nucleatum). In the present study there was no significant NANA-destroying activity in the cell extracts of the neuraminidase-positive B. fragilis NCTC9344 or in the cell extracts of the various cultures that gave low-positive or negative results in neuraminidase assays. Very few of these cell extracts reduced the assay value for added NANA by as much as 10-20% during incubation for 24 h under conditions equivalent to those of the neuraminidase

assay. This amount of NANA destruction would not appreciably reduce the ability to detect small amounts of neuraminidase activity. Several of the Capnocytophaga cell extracts produced a reduction of c. 40% in 24 h and this could have contributed to our difficulty in demonstrating neuraminidase production consistently in this group (Section Vd).

The results for neuraminidase production by Bacteroides species in the present studies are summarised in table Disc/III; they are consistent within each species and thus tend to confirm the present classification. Within the B. fragilis group, B. fragilis, B. vulgatus, B. distasonis and B. ovatus were all neuraminidase-positive. Seven strains of B. thetaiotaomicron were positive while six strains of the closely related B. eggerthi were negative. The B. variabilis strains were distinguished from the B. uniformis strains by their production of the enzyme, though it proved difficult to define conditions under which the enzyme was consistently produced by strain VPI11368 (table V/III).

Within the B. melaninogenicus - B. oralis group, B. oralis and B. melaninogenicus ss. melaninogenicus were neuraminidase-positive while ss. intermedius and B. ruminicola were negative; the single strain of B. melaninogenicus ss. levi that was available proved to be neuraminidase-positive. It has recently been suggested that the subspecies of B. melaninogenicus should now be regarded as separate species, and that strain ATCC15930 should become the type strain of a new species, B. loeschei (Holdeman & Johnson, 1982; Johnson & Holdeman, 1983). Strain VPI8906D, previously B. oralis but reclassified as the new species B. buccalis, produced the enzyme; strain NP333, previously B. ruminicola but

TABLE Disc/III

Production of neuraminidase by

Bacteroides species[a]

Neuraminidase-positive species	Neuraminidase-negative species
B. fragilis group	
B. fragilis (5)	B. eggerthi (6)
B. vulgatus (3)	B. uniformis (3)
B. distasonis (3)	B. splanchnicus (2)
B. ovatus (2)	
B. thetaiotaomicron (7)	
B. variabilis (2)	
B. melaninogenicus-B. oralis group	
B. melaninogenicus ss. melaninogenicus (3)	B. melaninogenicus ss. intermedius (5)
ss. levi (1)	B. pentosaceus (1)
B. oralis (3)	B. ruminicola (3)
B. buccalis (1)	B. disiens (2)
B. bivius (4)	
Asaccharolytic group	
	B. asaccharolyticus (3)
	B. gingivalis (1)
	B. corrodens (2)

[a] Extracted from tables V/III and IV; number of strains tested indicated in brackets.

now reclassified as B. pentosaceus, was neuraminidase-negative. The B. bivius strains produced the enzyme, while the closely related B. disiens did not. The strains of the asaccharolytic species B. asaccharolyticus, B. gingivalis and B. corrodens (B. ureolyticus) were all neuraminidase-negative.

Production of neuraminidase by Bacteroides species was studied by Muller & Werner (1970b; Werner & Muller, 1971) and reviewed by Hammann et al. (1981). These studies depended primarily on observing the electrophoretic changes in serum proteins in the culture medium after incubation of cultures for 2-19 days, but were also supported by paper chromatography of NAN-lactose and its split products. They found that all of 24 strains of B. fragilis produced the enzyme, but only six of 11 B. vulgatus, three of five B. distasonis and six of eight B. thetaiotaomicron could be shown to be positive. With our more direct assay procedures, using cell extracts of the cultures, all of the strains of these species examined were clearly shown to be neuraminidase-positive. At least one of their negative B. thetaiotaomicron strains (ATCC8492) is now reallocated to B. uniformis; this strain was negative in our assays also. Their findings with other Bacteroides species are in broad agreement with our present results: B. ovatus ATCC8483 and three strains of B. oralis (including ATCC15930, here classified as B. melaninogenicus ss. melaninogenicus) were positive, and single strains of B. asaccharolyticus (NCTC9337) and of B. putredinis were negative. In a later study (Marx et al., 1980) the same group of workers have also reported that a strain of B. bivius produces the enzyme.

The results of our tests in Bacteroides species are consistent and conform with the latest classifications (Holdeman et al., 1977; Duerden et al., 1980). They help to discriminate between several closely related species that are otherwise distinguished only by a small number of sugar fermentation reactions, e.g. between the species previously included in B. thetaiotaomicron, and between various species of the B. melaninogenicus - B. oralis group. It is possible that tests for neuraminidase production would be a valuable addition to the range of biochemical tests currently used in classification of Bacteroides organisms (see Collee et al., 1982, included in the Appendix).

The production of neuraminidase by other species of Gram-negative anaerobic bacilli was studied in Section Vd. Strains of Capnocytophaga ochracea were formerly considered as Bacteroides species but have been reclassified because they are CO_2-dependent rather than strict anaerobes (see Materials and Methods); the six strains tested could all produce the enzyme although it was difficult to define conditions that would give reliable production. Werner & Muller (1971) described neuraminidase production by one strain of Leptotrichia innominata var. ochracea, which would probably now be reclassified as Capnocytophaga ochracea (Hammann et al., 1981).

None of the Fusobacterium and Leptotrichia strains listed in table V/VI could be shown to produce the enzyme. Fukui et al. (1971) failed to detect neuraminidase in four strains of Fusobacterium species and two of Leptotrichia buccalis isolated from the mouth. However, Werner & Muller (1971) reported weak

neuraminidase activity against plasma proteins in two of ten strains of Sphaerophorus necrophorum (F. necrophorum), two of three S. varium (F. varium) including NCTC10560, and one of seven other Fusobacterium strains. Muller (1973b) isolated a strain of F. nucleatum from the throat of a patient with actinomycosis; there was strong neuraminidase activity on first isolation but this declined to a low level during subsequent laboratory passage. The Fusobacterium strains that we found to be neuraminidase-negative have been maintained in laboratory culture for some years and it remains possible that they might have lost the ability to produce the enzyme during storage. The taxonomy of the fusobacteria remains debatable (see Duerden et al., 1980) and little is known about their pathogenic potential or possible virulence factors.

B. fragilis is now well recognised as one of the most important Bacteroides species in clinical infections although the mechanism of pathogenicity remains poorly understood (see Ingham, Sisson & Selkon, 1980; Bartlett, 1982). The organism occurs as a minor component of the commensal anaerobic flora of the colon and vagina (Duerden, 1980a and b; Hammann, 1982) and causes infections predominantly when injury or damage to these mucosal surfaces allows access to otherwise sterile tissues. At one stage the B. fragilis group of organisms were regarded as subspecies of a single species, but they have now been reinstated as separate species on biochemical and genetic grounds (Cato & Johnson, 1976). Polk & Kasper (1977) confirmed the clinical importance of this distinction when they demonstrated that clinical isolates of these organisms from blood, wound and abscess cultures over a two-year period were predominantly B. fragilis (i.e. the former subspecies

fragilis); B. thetaiotaomicron, B. distasonis and B. vulgatus were much less commonly incriminated and B. ovatus was not encountered.

Polk & Kasper (1977) also noted that most B. fragilis strains were capsulate when freshly isolated from clinical material. Since only a small proportion of commensal strains are capsulate, interest has focused on the role of the capsule as a virulence factor promoting the selection and pathogenicity of these strains. In an experimental model of infection, implantation of faecal material leads to the development of peritonitis in rats; Escherichia coli may produce septicaemia and death during the first few days but the survivors develop chronic abscesses due to B. fragilis infection thereafter (see Bartlett, 1982). The relative roles of the two organisms were confirmed when gentamicin or clindamycin were used to inhibit either species selectively, and when pure cultures of the organisms were used in place of faecal contents. Only capsulate strains of B. fragilis cause infection in this model, and indeed injection of the purified capsular material itself may also lead to abscess formation (Onderdonk et al., 1977). It appears probable that surface components of B. fragilis impede phagocytosis and intracellular killing, though the precise mechanisms involved are debated (Ingham et al., 1981; Bartlett, 1982).

The roles of other factors in B. fragilis pathogenesis are still poorly understood. Intravenous injection of B. fragilis to experimental animals does not produce infection or toxic effects, perhaps because the endotoxin of the organism differs chemically from that of Escherichia coli and is not nearly so toxic (see

Bartlett, 1982). Werner and colleagues (Muller & Werner, 1970a; Hammann *et al.*, 1981) suggested that neuraminidase might play a role in the pathogenicity of *B. fragilis*, arguing that it is produced in greater amounts and has a broader spectrum of activity against a range of serum glycoproteins than the enzymes produced by other bacteroides species. Muller & Werner (1970a) studied pus from a *B. fragilis* abscess and showed that the enzyme was produced *in vivo* by demonstrating appropriate electrophoretic changes in the exudate glycoproteins; at present, however, there is no other evidence to support a significant role in pathogenesis. The enzyme is produced by all strains of *B. fragilis* examined, but it is also produced by the numerically dominant faecal species *B. thetaiotaomicron* and *B. vulgatus*. The pathogenic potential of these and other *Bacteroides* species is much less clearly understood at present; it will be interesting to note whether there is any correlation between their ability to produce neuraminidase and their occasional occurrence as pathogens.

STUDIES ON EXPERIMENTAL CLOSTRIDIAL MYONECROSIS

Experimental C. perfringens type A myonecrosis

The results presented in Section VIIa confirm the classical observations on experimental C. perfringens myonecrosis in the guinea pig that were described in the Introductory Review. C. perfringens strain L2Ab proved to be a virulent strain that could produce dramatic overwhelming infection with collapse and death within 28-48 h. There was marked local swelling with extensive necrosis and dissolution of the inoculated thigh muscles and accumulation of a large amount of haemorrhagic oedema fluid, often spreading up over the anterior abdominal wall. Microscopic examination showed many bacilli and few polymorphonuclear cells.

The virulence of strain L2Ab was assessed in experiments with graded challenge doses of washed log-phase cells. The minimum lethal dose was low ($<10^2$ cells) when initial muscle damage was produced by previous injection of $CaCl_2$ (table VII/I). In some control animals the injection of $CaCl_2$ itself produced a necrotic area that was demonstrable as a small sterile abscess when the animal was killed 6-7 days later; in others there was minimal transient swelling and stiffness but no abnormality was found post mortem. Larger doses (c. 10^6 organisms) were required for production of infection when adrenaline was used as initiating agent (table VII/II); there was no detectable abnormality in control animals given adrenaline without bacteria. Although these experiments were in general performed with a single animal for each challenge dose the results proved reasonably reproducible in later

experiments. It appears that the challenge dose required for establishment of infection is dependent on the degree and duration of hypoxia produced by the initiating procedure. The course of the disease in the two models was indistinguishable thereafter.

When sublethal challenge doses were given, infection might be established but it was usually contained locally and did not progress to produce the full picture of clostridial myonecrosis. The response to bacterial challenge was graded on the scale described in Materials and Methods so that the results could be presented succinctly in the tables in Section VII. It proved fairly easy to allocate each result to a particular grade although judgement of the precise distinction between, for example, small, medium and large abscesses, was subjective and might occasionally be debatable; the recorded results are certainly accurate to within one grade on this scale. Sublethal doses of strain L2Ab or other virulent strains usually produced a localised abscess in experiments with $CaCl_2$ as sclerosing agent. On occasion (e.g. table VII/III), the resultant abscess was little bigger than might be produced in some control animals given $CaCl_2$ alone, although it was clear from microscopic examination that the bacteria had multiplied considerably after inoculation. More frequently, however (e.g. tables VII/I, VI and VII), there was a medium or large abscess that was clearly due to bacterial infection. It appears that in this situation the host defences were adequate to limit extension of infection and prevent development of systemic illness; microscopic examination showed many polymorphonuclear cells and relatively few bacilli. When adrenaline was used as initiating agent, sublethal challenge doses occasionally produced

severe localised infection in the inoculated limb (e.g. table VII/IV) but more often there was little or no local reaction and no evidence of infection was found at post-mortem examination 6-7 days later (e.g. table VII/II). It seems that in these animals the challenge dose of organisms was insufficient to allow infection to be established before the vasoconstrictor effects of the adrenaline had worn off; thereafter the normal host defences were able to prevent damage and remove the organisms.

Various strains of C. perfringens type A were compared for virulence in guinea pigs with $CaCl_2$ as sclerosing agent (Section VIIc). After initial tests with a high challenge dose (c. 10^8 cells), a number of virulent strains were more fully assessed in tests with lower challenge doses. The types of infection produced in these experiments were similar to those seen with different challenge doses of strain L2Ab. A number of strains were clearly pathogenic, able to produce the full picture of clostridial myonecrosis and death within 24-48 h. Some variation was seen in different experiments but in general the results were reasonably consistent. Thus with the Hobbs` type-xviii strain, inoculation of challenge doses above c. $10^4$ cells produced severe infection with collapse or death before 48 h (table VII/VI). The results with the Hobbs` type-i strain suggested that the minimum lethal dose was c. 10^8 cells (tables VII/V and VII). An inoculum of c. 10^7 cells gave extensive local necrosis but the animal survived; it appeared that this strain might not prove lethal even when infection was rapidly established and progressed to give almost complete dissolution of the thigh muscles. The Hobbs` type-viii strain was investigated in a series of tests because a second experiment did

not confirm the initial finding that it could produce severe myonecrotic infection; however the final results, presented in table VII/VIII, demonstrated that high challenge doses of this strain could undoubtedly be fully pathogenic in this model.

Other strains proved unable to kill the animals even at high challenge doses although some could produce extensive local infection (table VII/V). Thus the animal inoculated with 10^8 cells of strain CW6 survived for 3 days even though only fragments of muscle remained in a huge accumulation of haemorrhagic fluid in the grossly swollen limb. With several other strains the animals survived for 6-7 days despite the production of large abscesses, which, however, remained localised in the inoculated muscle. Many other strains were clearly less virulent and even high challenge doses produced only small or medium-sized local abscesses, similar to those produced by sublethal doses of more virulent strains.

Although the full picture of lethal myonecrosis following administration of virulent C. perfringens has been well documented (e.g. see Willis, 1969, chap. 2), the range of effects produced by less virulent strains has not been so fully described. Evans (1943a), using an experimental protocol very similar to that in the present studies, reported results with four strains of the organism. Two strains produced lethal infection with challenge doses of 5-50 organisms; it appears that strain L2Ab is of comparable virulence. A third strain had a minimum lethal dose of 5×10^4 cells, but smaller doses produced "severe infection with survival". The fourth strain was not lethal with challenge doses of 5×10^7 cells, "although the animal become infected and exhibited well marked and typical lesions"; the grading system

used to record these lesions in his tables was not explained in detail but appears consistent with that used in the present study.

There is no absolute criterion of pathogenicity for C. perfringens strains. It is apparent that most strains are capable of multiplying and producing some degree of local infection when inoculated into an area of tissue damaged by prior injection of $CaCl_2$, but this can often be contained so that infection does not spread locally and there is no systemic illness. Strains that produce only this pattern of infection may be regarded as relatively avirulent and are presumably unlikely to produce serious clinical problems. A second group consists of strains that are able to cause severe local infection in damaged tissue, with extension of the local lesion to produce considerable muscle necrosis but without systemic collapse and death. In some cases the host defences can contain this within the muscle mass and a large localised abscess is formed; in others virtually the whole muscle mass is destroyed but the animal may survive for several days. A third group of strains contains clearly virulent organisms able to produce rapid and extensive local tissue necrosis and kill the animal in the acute phase of the illness. It is difficult to determine which is the critical level of virulence in this animal model that would be of significance in human infection; clinical experience during World Wars I and II might suggest that it is not unusual for environmental strains to prove virulent if introduced into a suitable wound. The apparent distinction between strains that do and do not produce death in experimental animals may reflect differences in degree rather than in kind, as the results of inoculation of high challenge doses of less virulent strains are

very similar to those obtained with sublethal doses of more virulent strains. The outcome of infection probably depends on a balance of factors, including the degree of tissue injury and the number of organisms achieved at an early stage of the disease as well as the virulence of the infecting strain as measured in this animal model.

The strains of C. perfringens type A tested for virulence in the present studies included classical and food-poisoning strains that were selected because of differences in various biological properties. Hauschild & Thatcher (1968) used a similar guinea-pig model to assess a selection of classical and food-poisoning strains. They found that the virulence of food-poisoning strains that were heat-sensitive and haemolytic was comparable to that of classical strains that were unrelated to food poisoning. In each group a number of strains were very virulent and able to produce myonecrotic infection with a low challenge dose, whereas other strains were less virulent, and some did not produce fatal infection even when 10^8 cells were injected. A few heat-sensitive but non-haemolytic food-poisoning strains were pathogenic when fairly high challenge doses were given, but none of the heat-resistant strains that they tested could produce gas gangrene. The results of the present studies with food-poisoning strains are included in table VII/IX, where the strains are ranked in approximate order of virulence; these findings are in general agreement with those of Hauschild & Thatcher (1968). A number of the heat-sensitive, haemolytic strains were fully virulent, able to produce the classical picture of clostridial myonecrosis (Hobbs' types viii, xvi, xvii and xviii); one heat-sensitive but

non-haemolytic strain (Hobbs' type i) was also pathogenic. The most virulent of the heat-resistant strains (Hobbs' type 21) produced a large localised abscess but did not kill the animal, and various other food-poisoning strains, both heat-sensitive and heat-resistant, also proved to be of lower virulence. It is clear that while heat-resistant food-poisoning strains may generally be of low virulence in the guinea pig model, the heat-sensitive strains may be able to produce either experimental gas gangrene or food-poisoning or both.

Table VII/IX shows the correlation between virulence and in-vitro production of α-toxin and θ-toxin for various strains of C. perfringens type A. Although α-toxin is also a haemolysin it is the θ-toxin that is responsible for the broad zone of complete haemolysis obtained on blood-agar media (see Materials and Methods); the terms haemolytic and non-haemolytic are used here to denote the presence or absence of θ-toxin activity. Production of α-toxin was judged by production of opacification around colonies on egg-yolk agar; this does not give an accurate measure of the maximum amount that a strain can produce in vitro but it allows strains to be divided into three groups - normal, low or negative (for discussion of assays for phospholipase-C, see Rigby, 1981). It should in any case be appreciated that the amounts of various toxins produced in vitro may vary considerably in different media (see Willis, 1969, chap. 2; Nord et al., 1974; Mollby et al., 1976). It is generally accepted that there is a reasonable correlation between α-toxin production and virulence for experimental animals, although Bullen (1970) has emphasised that discrepancies also occur (see Introductory Review). Willis (1969,

p. 122) has observed that "just as ten times the lethal dose of potassium cyanide is no more lethal than a single dose, so too a highly toxigenic strain ... is unlikely to cause effects more serious than those produced by a strain of minimum effective toxigenicity". Hauschild & Thatcher (1968) concluded that, for the classical and food-poisoning strains examined by them, their results "did not show a strict proportionality between virulence and (production of α-toxin)"; however a statistical correlation between the two factors was shown.

In the present studies, two virulent strains (Hobbs` viii and xvii) were consistently weak α-toxin producers, and two phospholipase-negative strains (CW6 and CW7) were able to produce severe local infection. Although these phospholipase-negative strains did not kill the animals during the acute infection they were originally isolated from human wound infections (see Materials and Methods); a further phospholipase-negative strain (NCTC11144) was a food-poisoning isolate (Pinegar & Stringer, 1977) and was found to be of low virulence in the guinea pig model. All three strains were confirmed to have remained phospholipase-negative when they were reisolated from the infected animal. Thus the present studies tend to confirm that there is not a strict correlation between α-toxin production and virulence; although the most virulent strains were phospholipase-positive, some moderately virulent strains might produce low levels or be phospholipase-negative, and many phospholipase-positive strains were of very low virulence. Most of the virulent strains were haemolytic, and most of the non-haemolytic strains were non-virulent (see table VII/IX). This probably reflects the generally low virulence of these unusual

food-poisoning strains rather than a direct link between θ-toxin production and virulence; it is unlikely that this oxygen-labile cytolysin will be active outside the area of established necrosis and there is little evidence to suggest that it plays a primary role in gas gangrene (see Introductory Review).

Food-poisoning strains of C. perfringens are generally regarded as "feebly toxigenic" because heat-resistant strains of the type initially incriminated in enteropathogenicity fail to produce the θ-haemolysin, and levels of α-toxin and other toxins are also usually low (Hobbs et al., 1953). Hauschild & Thatcher (1968) confirmed that non-haemolytic food-poisoning strains generally produce low levels of α-toxin, but found that heat-sensitive, haemolytic food-poisoning strains might produce quite high levels. Skjelkvale, Stringer & Smart (1979) re-examined the relationship between production of α-toxin and of enterotoxin with 239 strains of C. perfringens from a variety of sources. They showed that 86% of 65 strains that were associated with separate food-poisoning outbreaks produced enterotoxin in conditions that induced sporulation in vitro, whereas only two of 174 strains from environmental sources did so. Enterotoxin production was not confined to particular serotypes, and both positive and negative strains were found in the same serotype. In general, strains that were haemolytic produced higher levels of enterotoxin than those that did not produce θ-toxin, but there was no apparent correlation between the levels of enterotoxin and α-toxin produced; some high enterotoxin producers were low α-toxin producers and vice versa. Furthermore, they confirmed that five phospholipase-negative

strains that had been associated with food-poisoning outbreaks did produce enterotoxin.

Nakamura et al. (1976) examined strains of the non-haemolytic, phospholipase-negative species C. plagarum and demonstrated that on biochemical and genetic grounds they should be regarded as non-toxigenic variants of C. perfringens. Skjelkvale et al. (1979) confirmed that former C. plagarum strains were closely related biochemically and serologically to their phospholipase-negative food-poisoning strains, except that they were also enterotoxin-negative. Thus it appears that the species C. perfringens includes variants that do not produce one or more of these toxins (α-toxin, θ-toxin, enterotoxin). Strains that do not produce θ-toxin usually produce low levels of α-toxin but not infrequently produce high enough levels of enterotoxin to be involved in food-poisoning outbreaks. The common haemolytic strains may produce higher levels of α-toxin and of enterotoxin and may be implicated in gas gangrene or food poisoning. Strains that do not produce α-toxin are seldom isolated, perhaps partly because of the difficulties in recognising and identifying these organisms; however such strains may occasionally be associated with either wound infection or food poisoning.

The results presented in Section VIIb show that C. perfringens strain L2Ab produces neuraminidase in vivo during experimental myonecrotic infection in the guinea pig. With the present experimental procedure no enzyme activity was detected in normal guinea-pig liver or muscle. Tissue samples were disrupted by ultrasonic vibration for a few minutes and a tissue extract was prepared as described in Materials and Methods; this procedure was

presumably not sufficiently refined for extraction and detection of the relatively small amounts of neuraminidase that might be expected in lysosomal fractions of normal guinea-pig tissues (see Introductory Review). It was possible that muscle infection might by itself lead to the release of neuraminidase from host tissues; however no enzyme activity was demonstrable in abscesses produced by sterile $CaCl_2$ or by neuraminidase-negative organisms even when severe tissue damage was produced (see tables VII/III, V and X). The enzyme was shown to be present in large amounts in severe infections produced by strain L2Ab, whether $CaCl_2$ or adrenaline was used as initiating agent (tables VII/III and IV); variable amounts were also found in tissue samples from other sites, perhaps due to blood-borne spread of the enzyme but perhaps merely reflecting direct extension of infection. The enzyme was also present in the localised abscesses produced in response to injection of sublethal challenge doses (e.g. table VII/III).

Muller (1973b, 1974a) has suggested that strains of a number of bacterial species may lose the ability to produce the enzyme during laboratory subculture, and that it may be regained *in vivo*. However, no evidence was found that any of our neuraminidase-negative strains was induced to produce the enzyme during experimental guinea-pig infections (tables VII/V and VIII); this tends further to confirm the validity of our test procedures for defining neuraminidase-negative clostridial strains. The quantitation of neuraminidase present in infected tissues is not very accurate because the samples may vary from a portion of tissue containing a small localised abscess, but complete with surrounding fibrotic material and healthy muscle, to a sample of the

haemorrhagic oedema fluid formed when almost the entire muscle mass is destroyed. Nevertheless, neuraminidase was easily demonstrated in infections produced by neuraminidase-positive strains whereas none could be shown with neuraminidase-negative strains. In a number of cases, e.g. with the Hobbs' type-viii strain, it was confirmed that the organism remained neuraminidase-negative when it was reisolated from infected tissue. Thus, it is clear that the enzyme is produced *in vivo* by neuraminidase-positive strains and that strains defined as neuraminidase-negative *in vitro* remain so *in vivo*.

Muller (1970a) demonstrated electrophoretic changes indicative of neuraminidase action in exudate proteins from two patients with *C. perfringens* wound infections. Preliminary results (not reported here) have confirmed that the enzyme is produced *in vivo* during human infection. Neuraminidase was present in a sample of muscle tissue removed during surgical treatment of a diabetic patient who developed clostridial myonecrosis following a compound fracture of the leg. In this case two neuraminidase-positive clostridia (*C. perfringens* and a non-toxigenic *C. sordelli* strain) were isolated from the specimen as well as three neuraminidase-negative organisms (*C. sporogenes*, another phospholipase-negative *Clostridium* and *Bacillus coagulans*).

The results presented in table VII/IX indicate a general but imperfect correlation between neuraminidase production and virulence in the guinea pig model. Most virulent strains do produce the enzyme well but the virulent Hobbs' type-viii strain was neuraminidase-negative. Most of the other food-poisoning strains that did not produce the enzyme were of low virulence. The

pathogenicity of C. perfringens strains in producing experimental myonecrosis is likely to be multifactorial, as no single factor has been detected that is clearly correlated with virulence. In general, virulent strains produce a number of different products that might play a part in local tissue damage, e.g. α-toxin, θ-toxin, neuraminidase, but a few strains that appear to be at least moderately virulent and able to produce extensive local damage fail to produce one or other of these factors, and this makes it difficult to claim that production of any particular bacterial product is a prerequisite for the pathogenicity of C. perfringens type A.

Experimental myonecrosis with other clostridial species

Table VII/X contains the results of tests for pathogenicity in the guinea pig model with a variety of strains of other clostridial species. Although the total count of organisms was high (c. 10^7-10^8 cells), it is likely that with a number of the more oxygen-sensitive species there had been appreciable loss in viability in the challenge dose before injection into the animal; in a few cases a significant proportion of the inoculum consisted of spores (see Section VIId). Thus the results in table VII/X may tend to underestimate the virulence of some species by comparison with the results for C. perfringens strains in earlier tests.

The infection produced by C. septicum NCTC547 was very similar to that produced by C. perfringens strain L2Ab, with extensive local myonecrosis and death within 24 h. Aikat & Dible (1960) reported that the histological appearances of C. septicum

muscle damage were almost indistinguishable from those produced by C. perfringens. Tissue damage is attributed to the toxins produced by this organism (see Willis, 1969, chap. 4). The α-toxin is the major lethal toxin; it has haemolytic and dermonecrotic activity but is unrelated to C. perfringens α-toxin and its biochemical mode of action is unknown. C. septicum also produces a DNA-ase which may act as a leucocidin (β-toxin), a hyaluronidase (γ-toxin), an oxygen-labile haemolysin (δ-toxin) and a gelatinase. The haemagglutinin of C. septicum was studied by Gadalla & Collee (1967, 1968) and may be related to the neuraminidase, as discussed in the Introductory Review. The present studies confirm that C. septicum neuraminidase is produced in vivo (Gadalla & Collee, 1968; Muller, 1970b). The pathogenesis of C. septicum infections in man and animals has not been as intensively investigated as that of C. perfringens. Antiserum with high C. septicum anti-α-toxin gives passive protection against infection. It seems probable that the α-toxin plays the main role here, but that the other factors might also play a part in the local infection. There is some similarity in the disease pathology and in the potentially toxic factors produced by C. perfringens and C. septicum, and it is interesting to note that this extends to the production of large amounts of neuraminidase in muscle infected by either organism.

C. sordelli strain 1734 produced the typical picture associated with experimental infection by this organism, with swollen pink muscle, a gelatinous exudate and death within 48 h (Willis, 1969, chap. 5; Smith, 1975, chap. 15). Table VII/X shows that the other strains of this species produced abscesses of varying size, of the type seen with sublethal doses of

C. perfringens, rather than the typical C. sordelli reaction. Strain CB2 was quite virulent, however, with production of a large local abscess and death during the third day; strain P3 also produced extensive local infection with death on the fifth day, but the other strains, and the strain of C. bifermentans, produced smaller local lesions and did not kill the animals. The typical pathogenic effects of C. sordelli are attributed to its lethal and necrotising β-toxin, and it was confirmed that strain 1734 produced the toxin whereas the others did not. Toxigenic strains of C. sordelli are occasionally implicated in human and animal infections; non-toxigenic strains, and strains of C. bifermentans, are generally regarded as non-pathogenic contaminants (although many accounts refer simply to C. bifermentans, regarding C. sordelli as the same species and not reporting tests for toxin production or animal pathogenicity).

C. sordelli and C. bifermentans produce a number of other products that might be thought to be involved in pathogenesis; however, they evidently do not confer pathogenicity on C. bifermentans. Both species produce a phospholipase-C that has serological cross-reactions with the C. perfringens α-toxin but which has some differences in substrate specificity and is very much less toxic (Miles & Miles, 1947, 1950; Mollby, 1978); they have also been reported to produce an oxygen-labile haemolysin and some poorly characterised proteases (Willis, 1969, chap. 5). Arseculeratne, Panabokke & Wijesundera (1969) also reported the presence of DNA-ase and phospholipase-A activity and described a haemorrhagic toxin in cell extracts of a strain of C. sordelli. Mellanby & White (1968; White & Mellanby, 1969) separated these

components and confirmed that β-toxin, phospholipase-C, phospholipase-A, haemorrhagic toxin and neuraminidase were all distinct products in the cell extract; the neuraminidase was non-toxic in rabbit skin tests and did not enhance the toxic effect of β-toxin. The present results confirm that neuraminidase is produced *in vivo* during infection by *C. sordelli* but that none is produced by *C. bifermentans* (table VII/X). The finding that neuraminidase production is one of the few features that distinguish the two species raises the possibility that the enzyme may play a part in the pathogenicity of *C. sordelli*. It seems probable that the β-toxin is the critical factor that distinguishes very virulent strains of *C. sordelli*, but it may be that neuraminidase production contributes to the more limited pathogenicity of some non-toxigenic strains of *C. sordelli*, such as that found in these studies with the guinea pig model.

The infection produced by *C. novyi* NCTC538 was very similar to that produced by virulent *C. perfringens* and *C. septicum* strains (see table VII/X). A moderate challenge dose rapidly killed the animal, with extensive necrotic destruction of the inoculated thigh muscles; no neuraminidase was present in the infected tissues. The pathogenicity of *C. novyi* type A (see Willis, 1969, chap. 3) is attributed to its α-toxin, a lethal and necrotising toxin that has not been characterised biochemically. It also produces an oxygen-labile haemolysin (δ-toxin), a lipase (ε-toxin) and a phospholipase-C (ɣ-toxin) that is not related serologically to *C. perfringens* α-toxin and that differs in its substrate specificity and haemolytic reactions (see Oakley, Warrack & Clarke, 1947; Rutter & Collee, 1969; Taguchi & Ikezawa, 1975, 1977).

Boyd *et al.* (1972a and b) demonstrated that active or passive immunisation of sheep could prevent the development of gas gangrene with *C. novyi* spores implanted in gunshot wounds; as with *C. perfringens* infections, antitoxin was of little value in treatment of established infection. The sera used in these experiments were polyvalent, produced with toxoids prepared from products of *C. perfringens* and *C. septicum* as well as *C. novyi*; although the level of *C. novyi* anti-α-toxin was high, it is possible that antibody active against other *C. novyi* products might also have been present.

The present studies did not investigate the mechanisms of pathogenicity of the various clostridial species that may be associated with wound infection, but it is apparent that species that produce different toxins *in vitro* can produce similar myonecrotic infection in this guinea pig model and in clinical gas gangrene. As with *C. perfringens*, the injection of culture filtrates can reproduce most of the features of the local muscle damage (Aikat & Dible, 1956, 1960) and it is probable that combinations of the various toxic products and enzymes that have been identified are reponsible at least for initiation of the muscle damage and for many of the features of the local gangrenous infection. There is good evidence that toxins are important in a number of veterinary infections caused by related types of clostridia, e.g. enterotoxaemias and necrotic infections of muscle or liver. In these diseases infection may prove rapidly fatal; there may be a terminal septicaemia but death is often attributable to toxaemia with the major lethal toxin produced by the infecting organism. Stephen & Pietrowski (1981, pp. 74-5) emphasised the

dramatic success of active vaccination with clostridial toxoids in preventing a range of infections in sheep and cattle and conclude that "it is inconceivable not to ascribe an important role to C. perfringens β- and ε-toxins, C. novyi α-toxin (and) C. septicum α-toxin in the pathogenesis of (these diseases); however, one cannot absolutely rule out the possibility that other factors might also play a vital or ancillary role". Although consideration of these diseases may be thought to strengthen the general case for the importance of toxins in clostridial pathogenicity, Bullen (1970) was at pains to emphasise that the evidence was much less convincing for gas gangrene than for enterotoxaemic infections.

It is apparent that neuraminidase is produced both in vitro and in vivo by a number of important pathogenic clostridia and it may be generally true that virulent strains produce high levels of the enzyme, but it is clear that the correlation is not absolute. C. perfringens type A is the species most commonly incriminated in clinical gas gangrene, whether in war injuries or in civilian practice. The commoner involvement of C. perfringens might reflect a greater prevalence in soil, dust, etc., or its relative aerotolerance, allowing initiation of infection at higher Eh values, in less severely compromised tissue. C. septicum and C. novyi type A appear equally pathogenic and produce very similar experimental infection in guinea pigs, though C. novyi may have a longer incubation period in human infection (see MacLennan, 1962). Although it is tempting to postulate that the neuraminidase produced by C. perfringens and C. septicum might be a virulence factor for these organisms, it is striking that C. novyi, which can produce equally dramatic infection, does not produce the enzyme.

C. absonum and C. paraperfringens are regarded as non-pathogenic clostridial species that are related to C. perfringens in that they produce phospholipase-C enzymes that show serological cross-reactions with C. perfringens α-toxin. They proved to be of low virulence in the guinea pig tests (table VII/X). However some multiplication of bacteria occurred in the necrotic focus produced by $CaCl_2$ injection; neuraminidase was detected in the tissues of animals challenged with C. absonum but none was present with C. paraperfringens, and this conforms with the results of tests for production of the enzyme in vitro. Of the other neuraminidase-positive species, C. chauvoei is pathogenic for animals (blackquarter) but not for man, whereas C. tertium is regarded as non-pathogenic. C. tetani, C. botulinum and C. difficile, all neuraminidase-negative, are important non-invasive and essentially toxic human pathogens. C. sporogenes, which is closely related to C. botulinum, is neuraminidase-negative and essentially non-pathogenic, but C. histolyticum and C. fallax are neuraminidase-negative clostridia with invasive potential in experimental infections. The other clostridia found not to produce the enzyme in this study are regarded as non-pathogenic (see Willis, 1969, 1977). It is clear that there is no strict correlation between neuraminidase production and the ability of various clostridial species to cause wound infection; however, several pathogenic species produce neuraminidase in addition to recognised toxins and it is possible that this might contribute to muscle damage during infection by these organisms.

Protective effects of antisera in *C. perfringens* myonecrosis

A collection of antisera were characterised in preparation for studies to determine their protective effects in guinea pigs challenged with *C. perfringens* strain L2Ab. The standard assay for neuraminidase activity was adapted in order to demonstrate inhibition of the enzyme by antibody (Section VIa). The full picture of the inhibitory activity of an antiserum can be shown by measuring the effect produced by serial dilutions of the serum (e.g. fig. VI/2). For routine tests the titre of anti-neuraminidase activity was expressed as the percentage inhibition of the standard enzyme when tested at a dilution of 1 in 100; this avoided problems of non-specific enzyme inhibition due to glycoproteins in the undiluted sera. Although the slope of the curve might vary a little for different sera, perhaps reflecting variations in avidity of antibody, the degree of enzyme inhibition produced by the serum at a dilution of 1 in 100 gave a reasonable comparison of the anti-neuraminidase activity in the sera used in this study.

For the present studies it was judged to be more appropriate to measure antibody against neuraminidase by a direct assay that indicates inhibition of enzyme action than to use immunoassays that may be more convenient (e.g. in antigenic analysis of influenza viruses; see Introductory Review) but may measure antibody directed against other parts of the molecule. Few antisera were able to produce complete inhibition of enzyme activity even when tested undiluted. This problem may also arise with viral

neuraminidase, where it is attributed to the existence of separate antigenic and enzymic sites on the surface of the molecule, with enzyme inhibition occurring by steric hindrance rather than complete blocking of the active site (see Introductory Review). Published references to antibody against C. perfringens neuraminidase (e.g. Warren & Spearing, 1963; Nees & Schauer, 1974b) do not indicate the degree of inhibition achieved; in discussion at the conference at which the latter paper was presented Warren stated that "with Clostridium neuraminidase you only get 50% inhibition; it doesn't matter how much antibody is added" (Behring Institute Mitteilungen, 1974, 55, p. 149), although later in the discussion Nees (ibid., p. 154) said that their rabbit antiserum did give complete inhibition in vitro.

It is possible that there is antigenic variation between neuraminidases produced by different C. perfringens type A strains and that greater inhibition might be demonstrable in tests using the same neuraminidase preparation as was used for production of the antiserum. An indication that this might occur was obtained with the Rabbit-4 serum assays shown in figs VI/10 and 11; these suggested that antibody produced in response to the Sigma neuraminidase might appear more active when tested with that enzyme than with the standard strain L2Ab preparation (though the 1863 serum appeared to have similar activity against either enzyme). This was not explored further, since it appeared that assays for activity against the strain L2Ab enzyme would in any case be more relevant for studies designed to assess the protective effect of various antisera for animals that were to be challenged with that strain.

The specificity of anti-neuraminidase antibody in inhibition of enzymes from different sources has not been systematically investigated but the occurrence of antigenic shift and drift in influenza A viruses suggests that serological differences in the enzyme might occur even within closely related groups of micro-organisms. Weak cross-reactions have been observed between neuraminidases produced by different species within a single genus, e.g. with Streptococcus (Hayano et al., 1969), Pasteurella (Scharmann, 1974) and Arthrobacter (Huchzermeier, Tanenbaum & Flashner, 1980). Most studies have found no cross-reactions or cross-inhibition in comparative studies with antisera against enzymes produced by different genera of bacteria or by viruses or mammalian cells (e.g. Madoff, Eylar & Weinstein, 1960; Muller, 1974a; Scharmann, 1974; Huchzermeier et al., 1980). The exception to this was the early report by Warren & Spearing (1963) of serological cross-reactions involving the neuraminidases that they detected in semi-purified preparations of toxins produced by Corynebacterium diphtheriae and Clostridium perfringens. They showed that commercially available antitoxins prepared for use in clinical treatment of infections with either organism not only had anti-neuraminidase activity against the homologous enzyme but also could neutralise the enzyme produced by the other species. Studies in this laboratory (not reported here) suggested that one available sample of equine diphtheria antitoxin did inhibit C. perfringens neuraminidase, but it also had activity against C. perfringens phospholipase-C; a second diphtheria antitoxin preparation from a different source had no activity against either enzyme. It appears possible that equine antisera may contain antibody against a variety of bacterial

products that have been previously encountered by the animal as well as against the antigens currently being administered. The investigation was not taken further since it appeared unlikely that the reported cross-reaction between the enzymes of the two species would offer a means of obtaining an antiserum that would specifically inhibit neuraminidase action during experimental C. perfringens infections.

A variety of equine antisera against C. perfringens culture products were available in Prof. Collee's collection in this laboratory and were tested in order to assess their content of anti-neuraminidase (section VId). The results presented in fig. VI/2 show that the commercially available C. perfringens antitoxin used for demonstration of anti-phospholipase activity in diagnostic tests also had activity against neuraminidase, whereas the C. tetani antitoxin preparation could be used as a control equine serum with no anti-neuraminidase activity. The most potent of the equine antisera tested was an experimental serum (serum 1863) that had been prepared primarily for its anti-α-toxin activity and had been held in this laboratory for many years; it produced c. 80% inhibition when tested at 1 in 100 dilution in the standard anti-neuraminidase assay, and was used as reference serum in assays for titration of inhibitory activity in other sera (e.g. figs VI/2, 8, 10 and 12).

It appears from the results presented in Section VIc that C. perfringens neuraminidase is rather a poor antigen. Concentrated preparations of neuraminidase-containing culture supernate of strain L2Ab gave rise to very low levels of anti-neuraminidase activity in Rabbits 1 and 2 (fig. VI/4), although

analysis by gel diffusion showed that antibody had been produced to at least four other components in the crude antigen preparation (fig. VI/6). Three other rabbits were immunised with the purified Sigma enzyme preparation, with varied doses, adjuvants, routes of injection and immunisation schedules (table VI/IV). In each case there was a very slow rise in antibody titre that did not reach a maximum until 5-10 weeks after the initial injection and might remain at much the same level for many months thereafter (figs VI/7, 9 and 12). Further injections of antigen generally produced little booster effect, perhaps because the presence of circulating antibody impeded presentation of antigen to the immune system. The neuraminidase was initially given by the subcutaneous route with incomplete Freund`s adjuvant; however substitution of the complete Freund`s adjuvant, or the administration of larger doses of antigen by intraperitoneal or intravenous injection with aluminium hydroxide as adjuvant, still did not produce a satisfactory booster effect. The best effects were obtained in Rabbits 4 and 5, but the antibody levels achieved gave only 60-70% inhibition of the test enzyme in standard tests at a dilution of 1 in 100. Titration curves prepared with serial dilutions of these sera showed that the final serum from Rabbit 5 was the more potent of the two, with anti-neuraminidase activity equivalent to that in the reference equine serum 1863 (figs VI/10 and 13).

It is not clear why it is difficult to produce more potent anti-neuraminidase sera. The techniques used appear comparable to those employed in the other studies mentioned above in which antisera were raised against bacterial neuraminidases. It may be that our tests with antiserum diluted 1 in 100 are more demanding

than those used in other studies, but amounts of antibody are conventionally expressed in terms of serial dilutions of serum, and titres of antibody raised against other products are frequently very much higher than this. It seems unlikely that the poor response is attributable to the activity of the injected enzyme, e.g. on cells of the immune system, since the enzyme from myxoviruses appears to be a good antigen; some of the difference in response may be attributable to the particulate nature of the virus enzyme in contrast to the low MW of C. perfringens neuraminidase.

The anti-neuraminidase and anti-phospholipase titres of a range of equine and rabbit antisera were compared in standard assays (see table VI/V). Four equine antisera had high levels of anti-phospholipase but one other experimental serum and the C. tetani antitoxin had negligible activity; none of the rabbit antisera had activity against C. perfringens phospholipase. Serum 1863 and two other equine sera had fairly high levels of anti-neuraminidase activity, of the same order as that in the immune rabbit sera; the tetanus antitoxin and the pre-inoculation normal rabbit sera had insignificant activity. This collection of sera with different combinations of anti-neuraminidase and anti-phospholipase activity were used in animal studies in order to determine the correlation of protective effect and antibody content (Section VIIe).

Animals were challenged with C. perfringens strain L2Ab in doses close to the minimum lethal dose with either adrenaline or $CaCl_2$ as initiating agent (tables VII/XI and XII). The adrenaline model gave more clear-cut results; with some sera infection was

established and proceeded to produce myonecrosis, whereas with others there was complete protection or, at most, formation of a small localised abscess. With $CaCl_2$ as initiating agent, surviving animals developed more pronounced local infection in the damaged muscle; however, protection from lethal myonecrosis was clearly demonstrable in this model too. Bullen & Cushnie (1962) studied protection of guinea pigs challenged with a strain of C. perfringens that was described as feebly toxigenic but invasive and lethal; in their studies, protection was much more effective with adrenaline than with $CaCl_2$ as initiating agent. They used similar challenge doses in each model (27-65 x 10^3 cells with $CaCl_2$, 56-65 x 10^3 with adrenaline); an earlier report suggested that the minimum lethal dose for this strain with $CaCl_2$ was 50-100 cells (Bullen et al., 1961) and the use of a high challenge dose may have contributed to their difficulty in demonstrating protection with the $CaCl_2$ model.

In the present studies four equine sera (CPA2, 1863, 1881 and 2748) were clearly protective, whereas two others (5438 and ATS) were not; none of the sera from Rabbits 3, 4 or 5 were protective (tables VII/XI, XII and XIII). It is interesting that the same pattern of results was produced in each experimental model, as it is possible that the mechanisms involved might differ. Since adrenaline produces a transient ischaemic focus it is likely that the protective effect of serum in this model is related primarily to initial events important for establishment of infection during the first few hours. By contrast, $CaCl_2$ produces a long-lasting necrotic focus in which infection is easily established. The effect of antiserum in this model is not to prevent establishment

of infection but to control its progress so that the animal survives with infection localised in an abscess at the site of inoculation.

It is evident that the protective effect of the sera correlates with the level of anti-α-toxin, but not with the level of anti-neuraminidase (see table VI/V). The four protective equine antisera all had high anti-phospholipase activity whereas the two equine sera with low levels were non-protective; the rabbit sera contained no anti-phospholipase and were not protective (tables VII/XI, XII and XIII). Most of the equine sera with high anti-phospholipase activity also had high levels of anti-neuraminidase activity; however, serum 1881 was also protective despite its considerably lower anti-neuraminidase content. None of the immune rabbit sera were protective although they contained levels of anti-neuraminidase equivalent to those in the protective equine sera CPA2, 1863 and 2748. The effect of these sera appears to be comparable to that of the antisera studied by Evans and by Bullen in similar experimental models (see Introductory Review), though the present studies did not explore the fuller picture that would be obtained with graded doses of antisera, different challenge doses or alternative challenge strains of C. perfringens. Our results, therefore, tend to confirm those of Evans (1943a and b, 1945a, 1947), who concluded that the protection afforded by antisera in the early stages of experimental myonecrosis in guinea pigs is attributable to their anti-α-toxin content.

It is possible that the amounts of anti-neuraminidase present were inadequate to neutralise the enzyme in vivo despite their activity in vitro. Neuraminidase was produced in the infected

tissues and could be demonstrated in the animals challenged in the $CaCl_2$ model despite the presence of protective antisera; it was absent only in some animals given adrenaline where antiserum proved able to prevent infection completely (tables VII/XI and XII). The significance of this is not clear. The critical time for antibody action appears to be during the first few hours after challenge when small numbers of bacteria and small amounts of enzymes and toxins are present; the neuraminidase-positive samples from protected animals were taken 6 days later when a localised abscess had been produced by bacteria multiplying in the area of necrosis produced by injection of $CaCl_2$. The levels of anti-neuraminidase in the protective equine sera were as high as those that could be produced by immunisation of rabbits with purified enzyme, and it is difficult to suggest procedures that would be likely to produce higher levels. It might be possible to purify and concentrate anti-neuraminidase from these sera, e.g. by adsorption to immobilised neuraminidase, and it would be interesting to demonstrate whether increased amounts of anti-neuraminidase might have any protective effect. However, it is clear that the amounts of anti-neuraminidase present in protective antisera did not contribute to their protective effect.

CONCLUSION

The general case for considering that neuraminidase might have a role in bacterial pathogenicity is presented in the Introductory Review. Neuraminidase is produced *in vitro* by a number of important pathogenic bacteria. The enzyme is thought to have a nutritional role in nature and it is likely that it will also function in this way if it is produced *in vivo* during infection. Sialic acids are important constituents of circulating glycoproteins and of the surface membranes of a wide variety of fixed and circulating cells in man and animals. Mammalian neuraminidase is predominantly intracellular, and its activity is carefully controlled in the normal animal; decreased host enzyme levels are associated with disease, and it appears probable that the production of increased levels of neuraminidase during infection by bacteria that produce the enzyme *in vivo* will also upset delicate regulatory mechanisms in the host. The effects of treatment with bacterial neuraminidase have been studied with many different glycoconjugates and cell types; it is not directly toxic but can profoundly affect the normal distribution and lifespan of various glycoproteins and cells, and neuraminidase-induced antigenic changes in various cell types can lead to adsorption of naturally occurring antibody and cell damage. Neuraminidase might act as an aggressin, reducing surface charge and removing protective sialic acid from cells and molecules and facilitating further breakdown by other toxins and enzymes; it might have a particular role in attacking protective mucins overlying various epithelial surfaces in the body.

These possibilities were appreciated in outline by 1970. Intensive research on sialic acids in the last 10 years has sustained the general proposition that sialic acids are important molecules and hence that bacterial neuraminidase produced during infection might well contribute to host damage. It must be conceded, however, that little positive evidence for a pathogenic role for bacterial neuraminidase has been produced in this period. It has been suggested that there is a general correlation between virulence and production of the enzyme for a variety of pathogenic species, but there are many exceptions and it seems likely that the enzyme is also of value for various commensal organisms. The case for neuraminidase as a virulence factor for a variety of aerobic bacterial species was examined in the Introductory Review, but remains unsubstantiated.

The present studies have examined neuraminidase production by a variety of well characterised anaerobic bacteria in the genera Bacteroides and Clostridium. The taxonomy of the Bacteroidaceae has been much refined in recent years, with continuing alterations and subdivisions as the biochemical attributes of different species are defined. The present results are consistent within the species studied and it is suggested that tests for neuraminidase production might be of value in some aspects of classification of these organisms. Little is known of the pathogenic mechanisms of the Gram-negative anaerobes but it is clear that strains of B. fragilis, the species most commonly incriminated in infection, are strongly neuraminidase-positive in vitro and it is possible that the ability to produce the enzyme is a factor in the pathogenicity of this organism.

A number of pathogenic clostridial species are neuraminidase-positive, notably C. perfringens and C. septicum, two important organisms causing gas gangrene. Some C. perfringens strains do not produce the enzyme but most gangrene-producing strains do. Although there may be a general correlation between virulence and neuraminidase production in these gas gangrene species, the link is not absolute as C. novyi is equally virulent but neuraminidase-negative. Neuraminidase production is one of the few biochemical characters that distinguish the pathogenic species C. sordelli from the non-pathogenic C. bifermentans and this may be of value in taxonomic studies with these organisms. A variety of other clostridial species that produce different types of infections, e.g. C. botulinum, C. tetani and C. difficile, are neuraminidase-negative, as are many, but not all, of the saprophytic and commensal clostridia examined.

The pathogenesis of clostridial myonecrosis is discussed in the Introductory Review; attention has focused primarily on C. perfringens although the disease may also be produced by C. septicum and C. novyi, alone or in various combinations of the three species. Many of the features of the local muscle necrosis can be reproduced, in vitro and in vivo, by exposure of muscle to culture filtrates, and this supports the hypothesis that clostridial myonecrosis is attributable to the action of toxins and/or enzymes produced by these species. Experimental infection in the guinea pig reproduces many of the features of the human disease and has been used for elucidation of the role played by various toxic products. There may be a general correlation between virulence and α-toxin production by C. perfringens strains, and the

protective effects of antisera broadly reflect their content of anti-α-toxin. Several features of the disease, however, and notably the terminal toxaemic phase, do not appear to be adequately explained by theories that attribute the major role in C. perfringens pathogenicity to the α-toxin.

The present studies were designed to assess the hypothesis that neuraminidase might be an important factor in pathogenesis of clostridial myonecrosis. It was confirmed that the enzyme is produced in vivo during experimental infections by clostridial strains that produce the enzyme in vitro, and that no enzyme was detectable in infections produced by neuraminidase-negative strains. The enzyme was present in large amounts in the tissues of animals that died during fatal myonecrotic infection with C. perfringens or C. septicum but was absent from animals with comparable fatal myonecrosis produced by C. novyi. The enzyme was also demonstrable in samples taken from animals where the injection of non-pathogenic strains, or of sublethal doses of virulent strains, produced only a small localised infection.

Strains of C. perfringens that varied in their production of α-toxin, θ-toxin, enterotoxin and neuraminidase were selected and assessed for virulence in guinea pigs. These strains were naturally occurring strains isolated originally from a variety of sources including outbreaks of food poisoning, though they may not be representative of environmental strains liable to contaminate and infect wounds and injuries. It is difficult to determine criteria of virulence in experimental guinea-pig infection that will accurately reflect pathogenic potential in human wounds. Nevertheless, a number of strains were clearly virulent, able to

produce lethal experimental infection, while others produced severe local infection without causing death, and most strains could multiply to some extent in an area of damaged tissue, producing a degree of localised infection and abscess formation. There was no clear correlation between virulence and any of the bacterial products studied. The most virulent strains did produce α-toxin, although some apparently pathogenic strains produced rather small amounts, and at least one phospholipase-negative strain could produce severe local necrotic damage; however, the correlation between virulence and neuraminidase production was similarly incomplete, as at least one neuraminidase-negative strain was able to produce fatal myonecrotic infection.

A selection of antisera were characterised for their content of anti-α-toxin and anti-neuraminidase activity. A number of experimental equine sera had high anti-α-toxin titres and were able to protect animals challenged with a lethal dose of the virulent C. perfringens strain L2Ab. Several of these sera also had moderate anti-neuraminidase activity but rabbit antisera with equivalent amounts of anti-neuraminidase gave no protection. These studies tend to confirm the classical view that the protective effects of antisera in clostridial myonecrosis are attributable to their anti-α-toxin content. It appears that neuraminidase is rather a poor antigen and that it is difficult to produce antisera that will give good neutralisation of the enzyme when tested at high dilution; however, there is no evidence that anti-neuraminidase contributes to protection against experimental C. perfringens infection. The results of studies in guinea pigs can be related to human infection only with great caution, but the

limited protection given by antitoxic sera in the guinea pig appears to parallel clinical experience with antitoxin therapy in gas gangrene; there is no reason to suppose that improved protection in man could be achieved by increasing the anti-neuraminidase content of therapeutic antisera.

The present studies, designed to assess the relative importance of neuraminidase and α-toxin in experimental clostridial myonecrosis, have not explored other factors in pathogenesis, and little can be added to the general discussion in the Introductory Review. Infection is a dynamic process; the minimum lethal dose of a challenge strain is that which enables the organisms to multiply rapidly enough to outstrip the host's response and proceed to kill the animal. With sublethal doses the host is normally able to remove the bacteria and prevent infection. In experimental clostridial myonecrosis there is a delicate balance between host and bacterial factors and it appears that the initial few hours are critical in determining the eventual outcome of bacterial challenge. The degree and duration of tissue hypoxia is an important variable in the host's ability to respond. Infection is more easily established, with lower challenge doses, when a nidus of necrotic tissue is produced by injection of $CaCl_2$ than when transient ischaemia and reduction of Eh are produced with adrenaline. In either case, however, sublethal doses of the same virulent strain may produce infection that is contained locally and does not proceed to give the full picture of fatal myonecrosis. In this situation the outcome of infection does not depend on the biological properties of the strain but on the rate at which some critical point is reached; it appears that, after this point,

either the organism will proceed unchecked and overwhelm the host, or it will be contained and the host will survive. The fatal outcome is not dependent on the extent of local infection ultimately produced but rather on the initial rate at which infection is established.

The critical importance of the size of challenge dose might reflect the effectiveness of the host's phagocytic cell response. The difference in minimum lethal dose of C. perfringens with $CaCl_2$ or adrenaline used as initiating agent is attributable to different degrees of tissue compromise; ischaemia and Eh reduction may be important in impairing early phagocytic function in addition to their role in providing conditions suitable for clostridial growth. The strikingly low numbers of polymorphonuclear cells seen during acute infection suggests that bacterial products also interfere with the normal leucocyte response or have leucocidal activity. Differences in virulence of different challenge strains might reflect factors that confer protection against phagocytosis, e.g. the production of capsules (see Introductory Review). However, a correlation between virulence and capsule production has not been generally observed, and the absence of pus cells in acute infection suggests that the leucocidal effects of bacterial toxins may be more important in virulence. The present studies showed that many polymorphonuclear leucocytes were present when infection had been contained in a local abscess some days later; it might be instructive to compare the dynamics of the polymorphonuclear cell response in the initial stages of challenge with sublethal and lethal doses of various strains of C. perfringens. It may be that Bullen's studies, which emphasised the importance of non-specific

host defences involving serum factors, e.g. transferrin, have diverted attention from the role of the cellular response.

The protective effects of anti-α-toxin in the early stages of experimental infection with C. perfringens, though limited to situations where the degree of tissue damage is not too great, indicates that α-toxin plays a critical part in initiation of infection. This is particularly evident when infection follows an injection of washed cells mixed with adrenaline; early production of α-toxin is presumably required in order to damage cells and perpetuate low Eh values after the vasoconstrictor effects of the adrenaline wear off. The role of α-toxin in extending the local lesion is not so well attested, but it is not easy to ignore the evidence that α-toxin can by itself produce many of the features of muscle damage. The local infection is often rapidly progressive and no doubt there are two parallel processes, necrosis of tissues due to lack of oxygen and falling Eh and pH values, and active destruction of tissue by bacterial toxins and enzymes. The efficacy of hyperbaric oxygen and the lack of efficacy of antitoxic sera in preventing expansion of the lesion into neighbouring healthy tissue suggest that hypoxia is more important than α-toxin or other bacterial products at the edge of the infected area. Although C. perfringens may contribute to the extension of hypoxia by active removal of oxygen as suggested by Bullen, it appears unlikely that C. septicum or C. novyi, much stricter anaerobes, will play a comparable role; with infection by these organisms other factors, e.g. vascular damage, oedema, increased tissue pressure and shock, appear to be sufficient to produce rapidly extending muscle necrosis.

The present studies give little information about the mechanisms involved in the toxaemic phase beyond recording the fact of death following challenge with adequate doses of virulent organisms. There may well be differences between the processes leading to systemic collapse and death in man and in the guinea pig. Although death of a guinea pig during clostridial muscle infection is usually taken to indicate toxaemia, there might also be terminal septicaemic spread. Some guinea pigs survive despite massive muscle destruction and the accumulation of a large volume of haemorrhagic exudate around the hind limb; it is improbable that untreated patients would survive comparable damage and fluid loss, and it may be that guinea pigs are more resistant to shock than man. The speed of fluid loss from the circulation may be important in this model; perhaps a slower process of muscle destruction allows greater compensation. Modern techniques for investigation of mechanisms of shock and circulatory collapse could now give a much more detailed picture of the terminal stages of human gas gangrene than could be obtained in World War II. Fortunately, the disease remains rare in areas where such expertise is available, but such studies should be valuable in redefining the role of toxaemia in gas gangrene. It seems unlikely that a single toxin is the final lethal factor in clostridial myonecrosis, if only because there is no common bacterial product formed by C. perfringens, C. septicum and C. novyi that might be held responsible for systemic collapse and death; this, and the failure of antitoxic sera to prevent death, tend to suggest that more general mechanisms are involved.

The evidence against a major role for circulating C. perfringens α-toxin in the toxaemic phase of clinical gas gangrene is discussed in the Introductory Review. It is also unlikely that neuraminidase plays a role in the terminal stages of clostridial myonecrosis, but the possibility should be borne in mind. Haemolysis is a rare feature of the disease, and it might be attributed to a variety of mechanisms. However, T-activation of red cells has been detected on occasion during clostridial infections and it is possible that it is more frequently overlooked. It is not clear whether exposure of T antigen in vivo leads to significant red-cell damage by interaction with the patient's own natural anti-T, but haemolysis has on occasion been demonstrated in this situation following transfusion of whole blood containing anti-T; it may be safer to use packed cells where this risk may occur (see Introductory Review).

The results of the present studies leave C. perfringens neuraminidase in the same category as various other potential virulence factors, e.g. θ-toxin, hyaluronidase and collagenase of C. perfringens and equivalent products of C. septicum and C. novyi. The biological activities of these bacterial products suggest that they contribute to tissue damage and digestion in vivo. However, no strict correlation can be shown between ability to produce these factors and the virulence of different organisms, and antibody against them cannot be shown to contribute to protection against infection. It seems probable that these products do contribute to tissue digestion in the established lesion, and that the α-toxin also plays a critical role in initiation of infection. When anaerobic conditions are established the condition becomes

irreversible and none of the specific toxins plays an essential role in the progress of infection. Each pathogenic species produces a battery of cytolytic toxins and degradative enzymes that are of value to the organism in its commensal or saprophytic roles and it would be surprising if they did not also contribute to digestion of the tissues in an area where anaerobic conditions prevail, cells are dying and host defences are impaired; however, they do not appear to be essential for extension of the local lesion or the subsequent toxaemia, collapse and death. The detailed pathogenesis of gas gangrene is still not understood, but there is no evidence that neuraminidase plays a major role.

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APPENDIX

Parts of the work described in this thesis have already been published in the papers noted below. AGF was the main author of papers 1-5 but contributed only a portion of paper 6. Copies of the papers are appended.

1. FRASER, A.G. & SMITH, J.K. 1975. Preparation of a glycoprotein fraction from pooled human plasma and its evaluation as a substrate for the assay of Clostridium welchii (C. perfringens) neuraminidase. Journal of Medical Microbiology 8: 235-249.

2. FRASER, A.G. & COLLEE, J.G. 1975. The production of neuraminidase by food-poisoning strains of Clostridium welchii (C. perfringens). Journal of Medical Microbiology 8: 251-263.

3. FRASER, A.G. 1978. Neuraminidase production by clostridia. Journal of Medical Microbiology 11: 269-280.

4. FRASER, A.G. & COLLEE, J.G. 1979. Food poisoning caused by Clostridium perfringens (C. welchii) type A. Papua New Guinea Medical Journal (Focus Issue on Pig-Bel) 22: 87-97. (Note: unfortunately no proofs were made available before this article was printed.)

5. FRASER, A.G. & BROWN, R. 1981. Neuraminidase production by Bacteroidaceae. Journal of Medical Microbiology 14: 63-76.

6. COLLEE, J.G., BROWN, R., POXTON, I.R. & FRASER, A.G. 1982. Current approaches to the classification, characterisation and typing of pathogenic anaerobic bacteria. Scandinavian Journal of Infectious Diseases, Suppl. 35: 17-22.

PREPARATION OF A GLYCOPROTEIN FRACTION FROM POOLED HUMAN PLASMA AND ITS EVALUATION AS A SUBSTRATE FOR THE ASSAY OF *CLOSTRIDIUM WELCHII* (*C. PERFRINGENS*) NEURAMINIDASE

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PLATE XV

NEURAMINIDASES (EC 3.2.1.18) are widely distributed in nature, being found in viruses, bacteria, protozoa and vertebrate tissues. In mammalian cells, neuraminidase appears to be a lysosomal enzyme (Taha and Carubelli, 1967). The role of neuraminidase in the replication and pathogenicity of myxoviruses has been much studied but remains unclear (Drzeniek, 1972). Neuraminidase is produced by a number of important bacterial pathogens, e.g., *Clostridium welchii* (*C. perfringens*), *Corynebacterium diphtheriae*, *Vibrio cholerae* and *Streptococcus pneumoniae*, as well as by many harmless commensal organisms (Gottschalk and Bhargava, 1971). It has been suggested that neuraminidase production may be related to bacterial pathogenicity (Dewitt and Rowe, 1961; Collee, 1962 and 1965; Gadalla and Collee, 1968; Pardoe, 1970), but no positive correlation has so far been demonstrated (White and Mellanby, 1969; Drzeniek, Scharmann and Balke, 1972). In the course of investigations into the possible role of neuraminidase in infections caused by *C. welchii*, we found it necessary to develop a substrate for neuraminidase that is cheap and readily available in quantity.

Neuraminidases are usually assayed by measuring the release of N-acetyl neuraminic acid (NANA) from a suitable glycoprotein, glycolipid or oligosaccharide substrate. The free NANA can be measured in the presence of excess substrate by the thiobarbituric-acid assays of Aminoff (1959 and 1961) or Warren (1959 and 1963). Cassidy, Jourdian and Roseman (1965 and 1966) recommend the use of N-acetyl neuramin-lactose (NAN-lactose) prepared from bovine colostrum as substrate for the assay of *C. welchii* neuraminidase; this is widely used in neuraminidase assays, but commercially available preparations are very expensive, and it may be that the cost of NAN-lactose has been inhibiting further work on bacterial neuraminidases.

Popenoe and Drew (1957) prepared α_1-acid glycoprotein (orosomucoid) from the urine of nephrotic patients and showed it to be a suitable substrate for *C. welchii* neuraminidase assays, and Hughes and Jeanloz (1964) used α_1-acid glycoprotein prepared from human plasma as substrate in studies on pneumococcal neuraminidase. In early work on *C. welchii* neuraminidase in this laboratory (Collee, 1962 and 1965) hen egg-white was used as substrate.

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This was later replaced by human plasma (Gadalla and Collee, 1968), and Collee and Barr (1968) then described a simple method of preparing an unrefined glycoprotein fraction from 200–400 ml of plasma from out-dated human blood by trichloracetic-acid precipitation. This human glycoprotein gave more reliable results than were obtainable by the use of whole plasma, and it has proved a very satisfactory substrate in this laboratory. However, the limited supply of starting material and the problems of scaling up certain stages such as dialysis would make it difficult to ensure a supply of closely standardised substrate during a prolonged investigation.

In this paper we describe a method of preparing large amounts of a glycoprotein fraction from pooled human plasma. The α_1-acid glycoprotein is normally a waste product of large-scale plasma fractionation and a simple method of recovering it from the supernate from Cohn fraction V has been developed. It is considerably cheaper than commercially available NAN-lactose or pure α_1-acid glycoprotein, does not carry the risk of transmitting serum hepatitis (hepatitis B) and is recommended as a substrate for routine use in neuraminidase assays.

Materials and methods

Substrate preparations. The following method was used to prepare NANA-rich glycoprotein (fraction VII) from the supernate of Cohn fraction V, obtained from approximately 1000 donations of plasma from out-dated blood, by Cohn method 6 (Cohn *et al.*, 1946). A 120-litre volume of supernate from Cohn fraction V was titrated to *p*H 6·2 and cold (−25°C) ethanol added until the final concentration of ethanol was 70% (v/v); the temperature of the solution was lowered to −10°C during the addition, and maintained for 24 h. The resulting white precipitate was removed by continuous-flow centrifugation in a refrigerated Sharples Super-centrifuge at 20,000 *g* and a flow rate of 40 litres per h. Approximately 700 g of moist precipitate was recovered and dissolved rapidly in 5 litres water. The solution was adjusted to *p*H 8·0 with 2M NaOH and pasteurised in a stainless steel vessel at 60°C for 10 h. The solution was cooled, titrated to *p*H 5·0 with dilute acetic acid and held frozen at −20°C. After some weeks it was thawed at 5°C, centrifuged for 1 h at 4000 *g* to remove insoluble protein, and filtered rapidly through Green's 904½ fluted paper to remove floating lipoproteins. The solution was dispensed in vials, frozen and freeze-dried to a final vapour pressure of 13·33 N/m² (0·01 torr) at 20°C.

Two such large-scale preparations were made, differing in minor details of scale, resolution volume and the volume dispensed for freeze-drying; there was no detectable difference between the batches. The results given in this paper were all obtained with the original batch, FVII(5). Each batch was very readily soluble in water, even in concentrations exceeding 50 mg per ml.

Electrophoresis. Cellulose-acetate electrophoresis was carried out on Sepraphore III medium (Gelman Instrument Co., Ann Arbor, Mich., USA) at *p*H 8·6. Strips were stained for total protein (Ponceau S) and for glycoprotein (Kohn, 1968).

Total protein. Protein was estimated by a biuret method (Gornall, Bardawill and David, 1949); a copper-free control reagent was used to correct extinction readings for the opalescence of the glycoprotein preparations.

Electrolytes. Na^+ and K^+ were determined by flame photometry, chloride by a titrimetric method (Kit 830: Sigma Chemical Co. Ltd, Kingston upon Thames, Surrey) and Ca^{2+} by a fluorimetric method (Fingerhut, Poock and Miller, 1969).

Strain of C. welchii. This was a laboratory sub-strain (L2Ab) derived from a classical type-A strain originally obtained from Professor C. L. Oakley, School of Medicine, University of Leeds.

Culture media. Cultures, periodically prepared from lyophilised stock, were maintained

GLYCOPROTEIN SUBSTRATE FOR NEURAMINIDASE

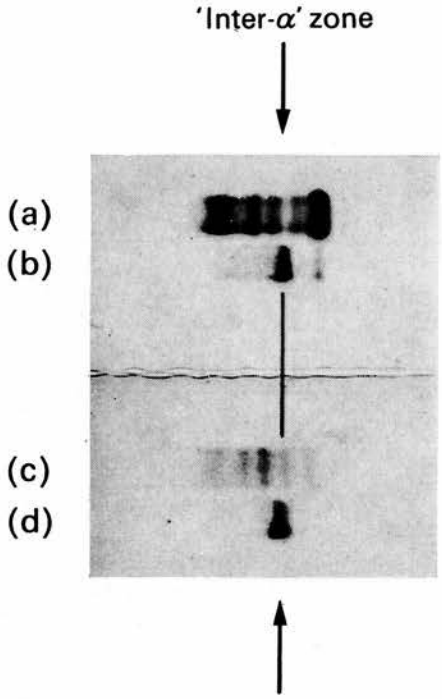

FIG. 1.—Separation of glycoprotein preparation FVII by cellulose-acetate electrophoresis. Samples (*a*) and (*c*), human serum; samples (*b*) and (*d*), fraction FVII. Strips (*a*) and (*b*) stained for total protein; strips (*c*) and (*d*) stained for glycoprotein.

in tubes (10 ml) of cooked-meat broth prepared as described by Cruickshank (1968, p. 757), but with Nutrient Broth (Oxoid, no. 2) replacing the peptone infusion broth.

The broth used for the production of neuraminidase contained (g per litre): Proteose Peptone (Difco, code 0120) 50 g and NaCl 5 g and was adjusted to *p*H 7·0. It was autoclaved at 121°C for 15 min. and is referred to as 5% proteose peptone water (PPW5).

Anaerobic culture. Broth media were held in a steamer at 100°C for 30 min. (pre-steamed) and promptly cooled to 37°C just before inoculation. BTL jars (Baird and Tatlock Ltd, Chadwell Heath, Essex) were used with room-temperature-active catalyst sachets supplied by BTL. The caps of bottles were loosened when they were put into anaerobic jars. Jars were filled with hydrogen and 10% (v/v) CO_2 according to the standardised procedure described by Collee, Rutter and Watt (1971).

Preparation of C. welchii neuraminidase. The test strain was inoculated into a tube of cooked-meat broth and incubated anaerobically at 37°C for 18 h; 1 ml of this culture was inoculated into 100 ml of pre-steamed PPW5 broth and incubated anaerobically at 37°C for 48 h; 20 ml of this starter culture was used as inoculum for each of three 600-ml (20-oz) narrow-mouthed Winchester bottles containing 500 ml of pre-steamed PPW5 broth which were then incubated anaerobically at 37°C for 48 h. The cultures were centrifuged at 1700 *g* for 1 h at 4°C and the culture supernates were pooled and stored at 4°C. The pooled supernate was passed through a Seitz filter and the filtered supernate (P9) was stored at −20°C for some months.

A 100-ml sample of P9 material was thawed and, after centrifugation (2400 *g* for 30 min. at 4°C) to remove a slight precipitate, the supernate was dialysed at 4°C in 25 mm (1 in) Visking Cellophane tubing for 72 h against running tap water, and finally against a large excess of distilled water for 24 h. This dialysed preparation (P9D2) had strong neuraminidase activity and was stored in 5-ml volumes in " bijou " bottles at −20°C. No phospholipase-C (α-toxin) activity was demonstrable in tube tests with egg yolk and calcium-gelatin-saline (Holding and Collee, 1971).

Neuraminidase standard. Our preparations of neuraminidase were standardised by comparison with a commercially available preparation of chromatographically purified *C. perfringens* (*C. welchii*) neuraminidase (Sigma London Chemical Co. Ltd; neuraminidase, type VI, N3001); the batch had a declared activity of 1·1 unit per mg of solid with NAN-lactose as substrate and 0·58 unit per mg with bovine submaxillary mucin as substrate, 1 unit liberating 1·0 μmole NANA per min. at *p*H 5·0 and 37°C from the test substrate.

Assay for N-acetyl neuraminic acid. Aminoff's thiobarbituric-acid assay for free sialic acid as described by Cassidy *et al.* (1966) was followed. The reagents were: *Reagent 1*—0·025M periodic acid (Koch Light) in 0·25M H_2SO_4 (BDH, Analar) at *p*H 1·2; *Reagent 2*—$NaAsO_2$ (BDH) 2% (w/v) in 0·5M HCl (BDH, Analar); *Reagent 3*—0·1M 2-thiobarbituric acid (Koch Light) in 0·1M NaOH (BDH, Analar) adjusted to *p*H 9·0; and *Reagent 4*—n-butanol containing 12M HCl 5% (v/v). Pure synthetic NANA (Koch Light) was used as a standard. In routine tests, 0·5-ml samples were assayed in glass tubes (125×15 mm). Spectrophotometric readings of the organic phase at a wavelength of 549 nm were made with 4-ml glass cuvettes (1-cm light path) in a Pye-Unicam SP 600 spectrophotometer. The reference cell contained a " reagent blank " prepared by taking 0·5 ml distilled water through the assay procedure.

Assay for neuraminidase activity. For the standard test, 0·5 ml of reaction mixture was prepared by adding the following volumes to glass tubes (125×15 mm): 0·1 ml of enzyme preparation; 0·15 ml of 0·1M sodium acetate buffer, *p*H 5·1, (Cruickshank, 1968, p. 853); and 0·25 ml of substrate FVII. Unless stated otherwise, dilutions of substrate or enzyme were made in acetate buffer, *p*H 5·1. Throughout the text the stated dilution of an enzyme preparation is that prepared for addition to the reaction mixture, but the concentration of substrate is normally given as the final concentration in the reaction mixture. Enzyme and substrate control tubes contained the equivalent volume of acetate buffer, *p*H 5·1, in place of the substrate and enzyme respectively. Reagents were normally held at room temperature before making the test mixtures, but were pre-warmed to 37°C when the period of incubation was to be less than 10 min. After incubation for the appropriate period at 37°C in

a water bath, reagent 1 was added to the mixture and the standard NANA assay was performed.

In studies of the time-course of the reaction with different concentrations of substrate and enzyme, standard tests were set up and incubated for graded periods of time. The timing of experiments was such that test periods of incubation ended simultaneously and all tests, including time-zero (T_0) control tests, were assayed for NANA in a single batch. The T_0 controls were set up as follows: the standard assay volumes of enzyme, substrate and buffer, pre-cooled to 0°–1°C, were mixed in a tube held in an ice bath at 0°–1°C. The assay for NANA was begun immediately by adding reagent 1 and transferring the mixture to a water bath at 37°C. As preliminary tests showed that the value for the T_0 control was equal to the sum of separate enzyme and substrate controls performed at T_0, and that the values for enzyme and substrate controls did not increase significantly during incubation for periods up to 24 h, a T_0 control was used in place of separate enzyme and substrate controls for each test period.

Each test and control assay was performed in duplicate and an average value for the extinction at 549 nm was calculated. The test reading was corrected to give a value equivalent to the amount of NANA released from the substrate during the incubation period by subtraction of either the sum of separate enzyme- and substrate-control readings or the value for the T_0 control test. Reference assays of 10 μg of pure NANA in 0·5 ml of distilled water were performed with each batch of assays. The results of neuraminidase assays are expressed in terms of spectrophotometric readings and are not routinely converted into equivalent concentrations of NANA because the presence of constituents of the culture medium may influence the reading obtained in the assay for NANA (see *Results*).

Studies of the effect of p*H on the neuraminidase assay.* Sodium-acetate buffer solutions were prepared as described by Cruickshank (1958, p. 853); the *p*H range was extended to give buffers of higher *p*H by the use of progressively smaller volumes of acetic acid and correspondingly larger volumes of sodium-acetate solution. In order to ensure adequate buffering, tests were performed in a greater volume than for standard neuraminidase assays. The total reaction-mixture volume of 1·0 ml consisted of: 0·1 ml of enzyme preparation P9D2 diluted 1 in 2 in distilled water; 0·65 ml of the appropriate buffer; and 0·25 ml of substrate FVII that had been diluted to 6·13 mg per ml in distilled water. Separate enzyme and substrate controls were prepared at each *p*H value by replacing the substrate and enzyme respectively with the equivalent volume of the appropriate buffer. Tests and controls were incubated for 30 min. at 37°C and then assayed for NANA by the standard method.

The starting *p*H values of this series of tests were measured during a separate experiment. With pre-cooled reagents, test mixtures were prepared in tubes held at 0°–1°C in an ice bath and the *p*H of each mixture was measured immediately. Small samples were brought to 37°C in a Radiometer BMS2 system and the *p*H values at 37°C were promptly read on a linked Radiometer PHM 71 meter. The *p*H values plotted for fig. 5 are these measured values for the initial *p*H of the reaction mixture. The tests were then incubated at 37°C for 30 min. and the final *p*H again measured. In no case was there a deviation of more than 0·05 *p*H unit during the assay period.

Tris-maleate buffer solutions were prepared as described by Diem and Lentner (1970); the *p*H range was extended to give buffers of lower *p*H by the use of progressively smaller quantities of NaOH. Tests were performed by the same method as for the tests in acetate buffer and initial *p*H values were similarly determined.

Tests for effects of culture materials on the NANA assay. The effects of various preparations on the NANA assay were investigated by preparing test mixtures of 0·1 ml of test substance, 0·15 ml of sodium acetate buffer, *p*H 5·1, and 0·25 ml of pure NANA in distilled water. Time-zero (T_0) tests were prepared with pre-cooled reagents. Each test value was corrected by subtraction of the value obtained for control mixtures with buffer in place of NANA. Reference tubes containing only NANA in buffer were prepared by replacing the test substance with buffer, and each test result was compared with the appropriate reference value to show whether the test substance had interfered with the NANA assay.

When it was desired to demonstrate the extent of NANA breakdown that might occur during incubation under the conditions of the neuraminidase assay, tests and controls were

also incubated at 37°C for the appropriate period before assaying for NANA. Experiments were timed so that test periods of incubation ended simultaneously and all tests, including T_0 tests, were assayed for NANA in a single batch. Any breakdown of NANA during incubation of a test mixture can be detected by comparing the corrected values for the incubated test and the T_0 test.

Time-course studies of the release of NANA from substrate by acid hydrolysis. Equal volumes (0·7 ml) of a known concentration of H_2SO_4 and of substrate dissolved in distilled water were mixed in screw-capped " bijou " bottles and promptly put into a water bath at 80°C. Reference bottles contained equal volumes of H_2SO_4 and of a solution of pure NANA in distilled water to demonstrate the extent of breakdown of NANA under the conditions of the test. The starting times were arranged so that the test periods of incubation ended simultaneously. The bottles were then rapidly cooled in two stages to 0°–1°C, duplicate 0·5-ml samples were transferred to tubes and the assay for NANA was immediately performed. Time-zero (T_0) control tests were performed with chilled reagents, the mixture being held for only a few min. at 0°–1°C before the assay for NANA. Test results were corrected by subtraction of the appropriate T_0 control readings.

RESULTS

Characterisation of the glycoprotein fraction FVII

A vial of the glycoprotein fraction FVII prepared as described in the *Methods* section contained 18·4 mg of dry powder. When this was dissolved in 1 ml of water, the solution contained protein 12·1 mg, Na^+ 46 μmole, $K^+ < 1$ μmole, Cl^- 8 μmole and Ca^{2+} 0·42 μmole.

Cellulose-acetate electrophoresis of fraction FVII (fig. 1) separated it into a minor zone with the mobility of serum albumin and a major, rather broad, " inter-α " zone with a mobility intermediate between the α_1 and α_2 serum globulins. The albumin zone stained very poorly for glycoprotein while the " inter-α " zone stained quite intensely. Because the relative dye-binding characteristics of these proteins were unknown it was not possible to assess the proportion of the total protein migrating in each zone.

Electrophoretic and immunoelectrophoretic examination of fractions of FVII separated on Sephadex G-200 confirmed that the " inter-α " zone consisted of α_1-acid glycoprotein, most of which had been polymerised during pasteurisation.

Release of NANA from the substrate FVII by hydrolysis

Release by acid hydrolysis. The total sialic acid present in fraction FVII was estimated by mild acid hydrolysis. When samples of it were heated at 80°C in 0·02M H_2SO_4 (final concentration) for periods up to 2 h there was progressive release of NANA, reaching a steady maximum between 1 and 2 h. When samples of NANA were similarly treated there was usually no significant breakdown and the extinction value obtained on assay of NANA incubated in acid for 2 h was used to convert the test value into the equivalent concentration of NANA. It was calculated that NANA constituted 4·7% of the dry weight of substrate FVII.

Release by C. welchii neuraminidase. The total amount of sialic acid that can be released from fraction FVII by *C. welchii* neuraminidase was determined

by incubation of dilutions of it with undiluted enzyme preparation P9D2 under the conditions of the standard test for periods up to 2 h. When reaction mixtures were incubated at 37°C in acetate buffer, *p*H 5·1, there was progressive release of NANA to a steady maximum after 80 min.; the same amount of NANA was released from our substrate by *C. welchii* neuraminidase as by the acid-hydrolysis procedure.

Enzyme-substrate kinetic studies

Initial velocity of neuraminidase reaction with varying concentrations of substrate. Enzyme preparation P9D2 was used at a dilution of 1 in 2 in the acetate buffer, *p*H 5·1, and a range of concentrations of substrate was prepared in the same buffer. Reaction mixtures were prepared for the standard neuraminidase assay and were incubated at 37°C for different periods of time before the NANA assay. Fig. 2 shows the plots of values for the amount of NANA released by the enzyme; these are linear for the first 10 min. for all test concentrations of substrate, and a value for the initial reaction-velocity at each substrate concentration was derived from this portion of the graph.

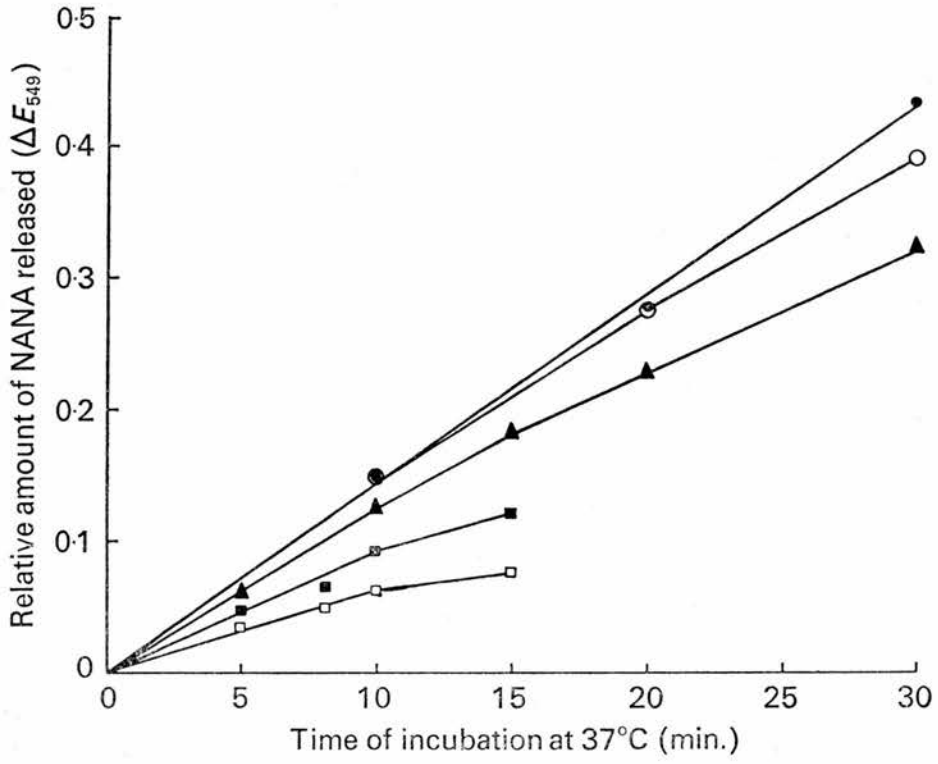

FIG. 2.—Velocity of neuraminidase reaction when a standard amount of enzyme P9D2 was incubated with graded concentrations of substrate at *p*H 5·1. Concentrations of substrate FVII (mg per ml of reaction mixture): ●——●, 9·2; ○——○, 3·07; ▲——▲, 0·92; ■——■, 0·23; □——□, 0·12. NANA = N-acetyl neuraminic acid.

Maximum velocity (*Vmax*) occurred at substrate concentrations of *c.* 3 mg per ml; the standard concentration of the original batch of substrate, FVII(5), used in subsequent experiments was 3·07 mg per ml. *Vmax* for this substrate was calculated to be 0·26 μg NANA per min. and the *Km* value was 0·16 mg per ml.

Initial velocity of neuraminidase reaction with varying concentrations of enzyme preparation. The final concentration of substrate FVII was 3·07 mg per ml in the reaction mixture, and enzyme preparation P9D2 was diluted in acetate buffer, *p*H 5·1, to give test concentrations of 10, 25, 50, 75 and 100% (v/v). Standard neuraminidase assays were carried out with the different

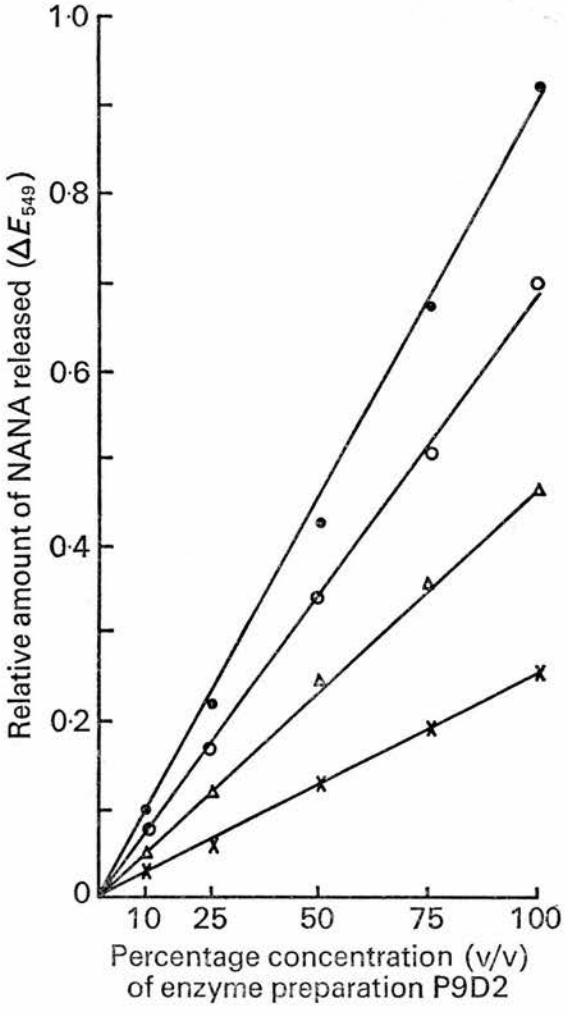

FIG. 3.—Amounts of NANA released at different times from a standard amount of substrate FVII by graded amounts of *C. welchii* neuraminidase. Period of incubation at 37°C (min.): ●——●, 40; ○——○, 30; △——△, 20; ×——×, 10.

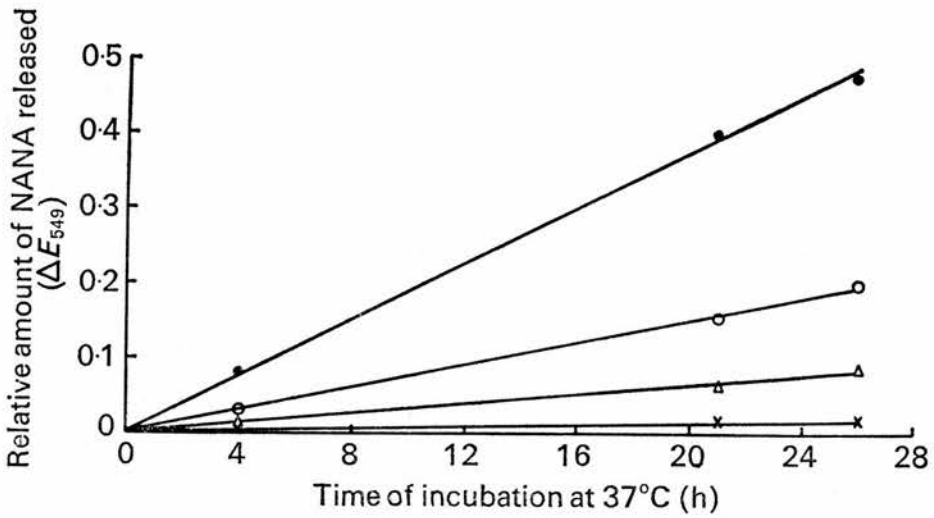

FIG. 4.—Release of NANA from substrate FVII by very low concentrations of neuraminidase during prolonged incubation at 37°C and *p*H 5·1. Percentage concentration (v/v) of enzyme preparation P9D2 added to reaction mixture: ●——●, 2; ○——○, 1; △——△, 0·5; ×——×, 0·1.

reaction mixtures incubated at 37°C for 10, 20, 30 or 40 min. before the NANA assay. In fig. 3 the rate of release of NANA is shown to be proportional to the concentration of the neuraminidase preparation when the reaction mixtures were incubated for periods of up to 40 min. Thus, using a 15-min. assay, we could measure amounts of neuraminidase approximately 0·1–3 times that of the test P9D2 preparation. More concentrated neuraminidase preparations should be diluted before the assay so that the results fall into the reliable range.

Detection of release of NANA by small amounts of neuraminidase after prolonged incubation with substrate FVII. Experiments similar to that described above were used to follow the release of NANA from the same amount of substrate by lower concentrations of enzyme P9D2 during longer periods of incubation at 37°C. Fig. 4 shows that the plot of the release of NANA from the substrate is linear for 26 h with suitable dilutions of enzyme P9D2 and that as little as 0·5% of the enzymatic activity can be reliably detected with a 24-h test.

The effect of pH on the neuraminidase assay. The results of the neuraminidase assay at different *p*H values in acetate buffer are shown in fig. 5(*a*). The plot of uncorrected results of duplicate tests shows a major peak of activity around *p*H 4·6 and a subsidiary peak around *p*H 5·6. The results of single-tube substrate-control assays are also plotted and these suggest that the main peak is actually at *p*H 4·7. Fig. 5(*b*) shows the results of a similar experiment when tris-maleate buffers were used. There is a single peak of activity at *p*H 5·7.

Direct readings of the initial and final *p*H of standard neuraminidase assays of dialysed and undialysed preparations of culture filtrate P9 in acetate buffer, *p*H 5·1, showed that the initial *p*H of the reaction mixtures might be as high as *p*H 5·45, but did not vary during incubation.

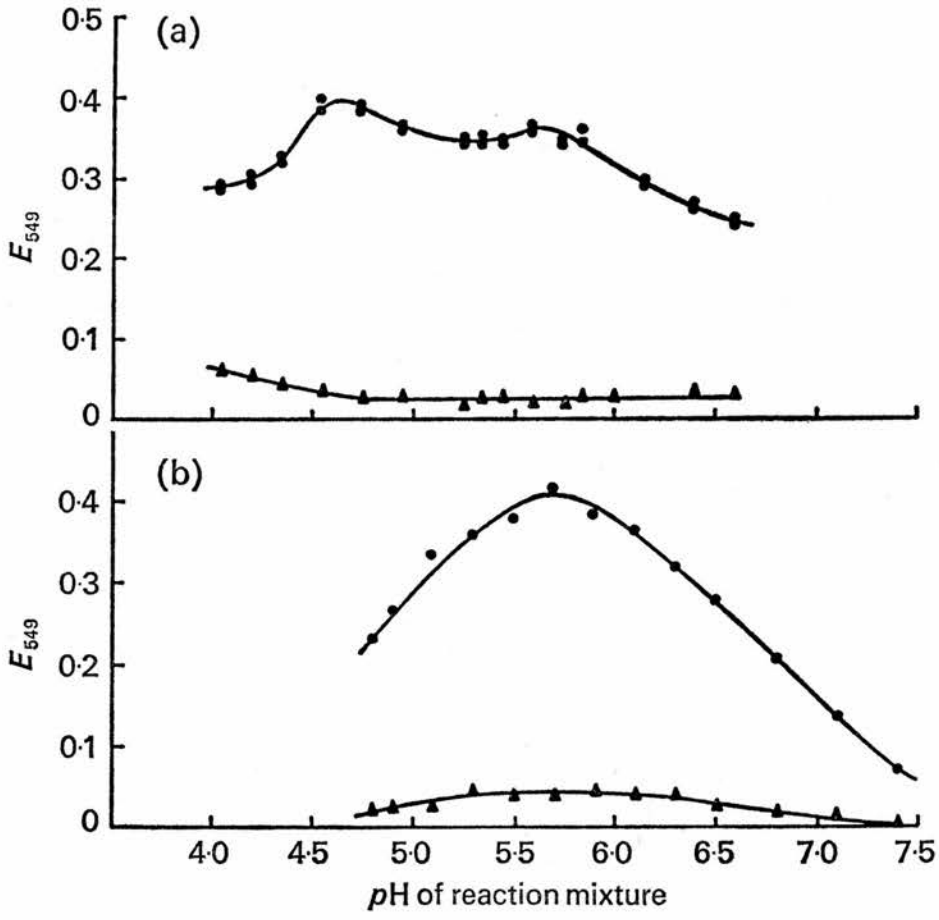

FIG. 5.—Effect of *p*H and buffer system on the neuraminidase assay. Enzyme preparation P9D2 incubated with substrate FVII, (*a*) in sodium-acetate buffers, (*b*) in Tris-maleate buffers. Uncorrected test readings, ●——●; substrate-control readings, ▲——▲. The enzyme control readings were very low and are not plotted.

Effects of culture materials on the NANA assay

When a sample of sterile PPW5 broth is taken through the NANA assay the formation of a chromogen with a peak absorption at 530 nm gives rise to significant absorption at 549 nm. This gives rather high enzyme control values in assays of supernates of cultures in this medium, e.g. P9; the effect is very much reduced after dialysis.

When a standard amount of NANA is assayed with PPW5 culture supernate in the assay mixture, the corrected value (after subtraction of the enzyme-control value) is sometimes markedly less than the value obtained in an equivalent assay devoid of culture supernate. The effect may also be seen with sterile PPW5 broth or other culture media; it is most marked with test mixtures that have high enzyme control readings (see below).

Tests for destruction of NANA under the conditions of the neuraminidase assay

Samples of pure NANA (10μg) were incubated for periods up to 60 min. with sterile PPW5 broth or with enzyme preparations P9 or P9D2 under conditions equivalent to those of the neuraminidase assay. The corrected value for the time-zero (T_0) assay of each test mixture showed the extent of depression of the true value for NANA before the start of incubation at 37°C. Sterile PPW5 broth reduced the assay reading by about 10% and the undialysed P9 preparation reduced it by about 25%, whereas the dialysed preparation P9D2 produced less than 5% depression of the assay reading. There was no significant further depression of the assay values when the mixtures were incubated at 37°C for 60 min., and there is therefore no evidence of significant N-acyl neuraminic acid-aldolase (NAN-aldolase: N-acyl neuraminate lyase, EC4.1.3.3) activity in either of the test enzyme preparations under the neuraminidase assay conditions. However, in view of the marked depression of the true NANA level by undialysed culture fluids, it is recommended that dialysed preparations be used where possible.

Comparative studies on neuraminidase preparations

Samples of Sigma neuraminidase in distilled water were compared in our assay with undiluted samples of undialysed enzyme preparation P9 and the dialysed preparation P9D2. The 0.1-ml enzyme samples were incubated for 15 min. at 37°C, *p*H 5·1, with substrate FVII at a final concentration of 3·07 mg per ml in standard 0·5-ml reaction mixtures. Separate enzyme and substrate controls were included and the results are shown in the table. The corrected spectrophotometric reading for undialysed enzyme P9, which has a high enzyme-control reading, is a minimum value because interfering substances in the culture supernate must be assumed to have depressed the assay; the dialysed preparation has a satisfactorily low control value. The Sigma neuraminidase

TABLE

Results of 15-min. neuraminidase assay of Clostridium welchii enzyme preparations

Test enzyme preparation*	Spectrophotometric reading (E_{549})†			Neuraminidase activity§ (milliunits per ml)
	Test	Enzyme control	Corrected value‡	
Sigma	0·614	0·000	0·589	27·5
P9	0·729	0·174	0·530	24·7
P9D2	0·454	0·015	0·414	19·3

* Sigma = chromatographically purified neuraminidase (Sigma Chemical Co., Ltd) at an initial concentration of 25 μg per ml (0·1 ml of this solution used in assay); P9 = undialysed supernate of culture of *C. welchii* strain L2Ab in PPW5 broth; P9D2 = dialysed sample of P9.

† Average of duplicate readings.

‡ Test reading corrected by subtraction of the sum of the values for the substrate control (0·025) and the corresponding enzyme control.

§ See text for method of calculation.

was stated by the manufacturers to have a potency of 1·1 unit per mg of solid when tested with NAN-lactose as substrate; the concentration of Sigma neuraminidase in the test solution would thus have an activity of 27·5 milliunits per ml. Separate tests similar to those described in the preceding section showed that this concentration of Sigma neuraminidase produced no depression of the assay value for NANA and no reduction in reading during incubation at 37°C for 15 min. The corrected test value of E_{549}=0·589 for Sigma neuraminidase can thus be used to calculate the approximate potency of our neuraminidase preparations in terms of the standard unit based on the release of NANA from NAN-lactose.

The reference sample of 10 μg of pure NANA gave a reading of 0·513 in this experiment. This allows calculation of the rate of NANA release from substrate FVII by the test amount (2·5 μg) of Sigma neuraminidase; thus the potency of this batch of Sigma neuraminidase when acting on our substrate is approximately 1·0 unit per mg of solid, as compared with the stated 1·1 unit per mg with NAN-lactose or 0·58 unit per mg with bovine submaxillary mucin as substrate.

Discussion

Most of the glycoprotein in our preparation was polymerised, probably as a result of the pasteurisation procedure (Spragg *et al.*, 1969). This partial denaturation may facilitate hydrolysis of the substrate by neuraminidase. Although the risk of transmission of serum hepatitis by the fraction that we use is relatively small (Berg *et al.*, 1972), it would be unwise to use the unpasteurised product in normal laboratory conditions. The glycoprotein fraction described by Gadalla and Collee (1968) is also liable to contain the agent of serum hepatitis, although the risks are low because plasma from only a few donors is used. If that preparation is being used, great care should be taken in handling the plasma and it should be pasteurised (60°C for 10 h) at some stage during preparation.

The pasteurised FVII preparation is not an ideal substrate in that it does not consist of a pure, well-characterised substance, but it possesses certain compensating advantages. It is produced from a "waste" fraction with few manipulations and can therefore be made available comparatively cheaply. It is easy to prepare in standard form in large quantities and is very stable as a freeze-dried powder and even in solution at 4°C. Its preparation does not involve the use of zinc salts (Bezkorovainy and Winzler, 1961), the residues of which might inhibit the action of enzymes on the substrate. The preparation has a low ionic content, the only residual buffer being a small amount of acetate, and its *p*H and ionic strength can therefore be easily adjusted in any incubation mixture. The content of readily hydrolysable NANA is high enough to avoid problems of viscosity or turbidity in the incubation mixture, and the level of free chromogen before hydrolysis is conveniently low. It must be borne in mind, however, that the preparation may contain a mixture of glycoproteins that might be hydrolysed at different rates by the same or by different enzymes.

Cassidy *et al.* (1965) found that the NANA in α_1-acid glycoprotein could be completely released by *C. welchii* neuraminidase and that the initial rate of release was slightly faster than the initial rate of release from bovine NAN-lactose. About 80% of the NAN-lactose in bovine colostrum is N-acetyl neuramin-(2→3)-lactose but the (2→6) isomer is also present (Schneir and Rafelson, 1966; Drzeniek, 1973). Viral neuraminidases may show a marked difference in ability to hydrolyse (2→3) and (2→6) linkages; both linkages are completely hydrolysed by *C. welchii* or *V. cholerae* neuraminidase although the rate of the reaction is rather slower with the (2→6) isomer (Cassidy *et al.*, 1965; Drzeniek, 1967). In α_1-acid glycoprotein, NANA is linked to the carbohydrate side chains by both (2→3) and (2→6) linkages (Drzeniek, 1973).

A variety of acyl groups are found on the neuraminic acid of naturally occurring compounds. Since the Warren and Aminoff assays give lower readings for N-glycolyl than for N-acetyl derivatives (Warren, 1959; Aminoff, 1961; Drzeniek, 1972), it is desirable that a substrate should contain only the N-acetyl derivatives. Human α_1-acid glycoprotein, unlike that from many other species, does not contain N-glycolyl neuraminic acid (see Jeanloz, 1972). It has generally been considered that human tissues contain only the N-acetyl derivatives (Gottschalk, 1960), but Walkowiak, Kedzierska and Starzynski (1968) have reported a trace of N-glycolyl neuraminic acid in human serum.

The α_1-acid glycoprotein prepared from nephrotic urine was shown to be a good substrate for assays of *C. welchii* neuraminidase by Popenoe and Drew (1957) and we have now shown that our FVII preparation from human plasma is also a good substrate. The calculations based on the activity of purified *C. welchii* (Sigma) neuraminidase in our assay sugggest that the initial velocity of release of NANA from our substrate is comparable to that from bovine NAN-lactose. The assay procedure that we describe allows accurate measurement of a wide range of concentrations of the enzyme. Even very small amounts of neuraminidase activity can be detected with confidence by prolonging the period of incubation to 24 h, and this is of value when it is important to demonstrate the absence of neuraminidase, e.g. in comparative studies of neuraminidase production by different strains of *C. welchii* (Fraser and Collee, 1975).

Varying the *p*H of our assay in tris-maleate buffer produced a plot of activity that showed a simple curve with a clear-cut optimum at about *p*H 5·7. When sodium-acetate buffer was used, the curve was more complex with the main peak at about *p*H 4·7 and a subsidiary peak at about *p*H 5·6. The *p*H optimum for the activity of a particular neuraminidase may vary considerably when tested with different substrates (Rafelson, Schneir and Wilson, 1963) or in the presence of different buffer ions (Cassidy *et al.*, 1965). Burton (1963) found a sharp *p*H optimum at *p*H 5·0–5·1 for *C. welchii* neuraminidase tested with a ganglioside substrate in acetate buffer. Popenoe and Drew (1957) found the optimum to be *p*H 5·0–5·5 in acetate buffer with α_1-acid glycoprotein as substrate. Cassidy *et al.* (1965) found the optimum for *C. welchii* neuraminidase acting on bovine NAN-lactose to be approximately *p*H 5·6 in tris-maleate or in citrate-phosphate buffers but between *p*H 4·0 and 4·8 in acetate buffer. Our finding of a subsidiary peak around *p*H 5·6 in acetate buffer with fraction FVII as substrate may reflect a heterogeneity of NANA linkages in the preparation, perhaps due to the presence of a mixture of glycoproteins or to alterations in glycoproteins produced by pasteurisation; it might also be due to different *p*H optima for the hydrolysis of (2→3) and (2→6) linkages, as was found for influenza-virus neuraminidase by Schneir and Rafelson (1966). Cassidy *et al.* (1966) recommend that the assay of *C. welchii* neuraminidase with NAN-lactose as substrate should be performed in potassium-acetate buffer at *p*H 4·5. When the assay is performed with FVII substrate and sodium acetate buffer at *p*H 5·1, the results fall on the high plateau between the two peaks of activity; for assays performed in tris-maleate buffer the *p*H should be *p*H 5·7 with this substrate. *C. welchii* neuraminidase does not require added Ca^{2+} (Cassidy *et al.*, 1965); optimum buffer and ion conditions may differ when fraction FVII is used as substrate for enzymes derived from other organisms or from viruses.

C. welchii produces neuraminidase well when grown in PPW5 broth,

and the culture filtrate is a good source of neuraminidase. When undialysed culture filtrate is assayed, there is a rather high control value in the neuraminidase assay, and we have found that there may be a significant depression of the assay value for pure NANA. Different batches of PPW5 broth vary in the extent to which they depress the NANA assay and the 25% depression produced by our culture filtrate P9 is the most marked that we have observed. The effect is minimal with dialysed preparations, e.g. P9D2, or with purified (Sigma) neuraminidase. We have not investigated the mechanism of the depression of the NANA assay by dialysable components of the PPW5 broth; a number of substances are known to interfere with the thiobarbituric acid assay for NANA (see Tuppy and Gottschalk, 1972) and this should be taken into account when neuraminidase assays are performed on unpurified enzyme preparations.

C. welchii also produces N-acyl neuraminic acid-aldolase (NAN-aldolase), an enzyme that hydrolyses NANA to N-acetyl mannosamine and pyruvate (Comb and Roseman, 1960). This enzyme is normally intracellular, but Popenoe and Drew (1957) found a little activity in culture filtrates of *C. welchii*; however, our enzyme preparations did not reduce the assay value for NANA during incubation under conditions equivalent to those of the neuraminidase assay.

Neuraminidase activity is commonly expressed in terms of units; 1 unit liberates 1 μmole of NANA per min. from the substrate at 37°C in a suitable buffer at the optimum *p*H (Drzeniek, 1972). It seems desirable that neuraminidase activities should be stated in standard units measured with NAN-(2→3)-lactose as substrate and we have refrained from expressing our results in terms of units measured with our substrate, though we have related the potencies of our test enzyme preparations to a commercially available reference preparation (Sigma neuraminidase). We have shown that our product is a good substrate for routine use in assays of neuraminidase from *C. welchii*, and preliminary experiments suggest that it should also prove valuable in studies of neuraminidase from other sources, e.g. *Corynebacterium diphtheriae* (personal communication, Dr D. C. Edwards and Mrs Karen Fidgen, Wellcome Research Laboratories, Beckenham, 1972), *V. cholerae*, influenza virus and Newcastle disease virus. It is hoped that the availability of this substrate at reasonable cost will encourage further studies on bacterial neuraminidases.

Summary

A glycoprotein fraction (fraction VII) suitable for use as a substrate in assays of microbial neuraminidase was prepared from pooled human plasma. It is pasteurised during preparation to eliminate the risk of transmission of serum hepatitis. This results in polymerisation of some of the α_1-acid glycoprotein, but fraction VII is shown to be an excellent substrate for the neuraminidase of *Clostridium welchii* (*C. perfringens*). A sensitive assay procedure is described. A number of factors may interfere with the measurement of NANA released by the action of microbial neuraminidase and procedures

are described for evaluation of this problem. Fraction VII is easy to prepare, cheap and available in standard form in large amounts (inquiries should be addressed to J. K. S.); it is recommended for routine use as a convenient substrate for neuraminidase assays.

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THE PRODUCTION OF NEURAMINIDASE BY FOOD-POISONING STRAINS OF *CLOSTRIDIUM WELCHII* (*C. PERFRINGENS*)

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THERE is confusion in the literature regarding the production of neuraminidase (EC 3.2.1.18) by food-poisoning strains of *Clostridium welchii* (*C. perfringens*) type A; this partly reflects the changing definition of these food-poisoning strains. Many episodes of food poisoning caused by *C. welchii* result from ingestion of strains that produce very heat-resistant spores, i.e., spores that survive boiling in faecal suspension for 1 h or more (Hobbs *et al.*, 1953). These strains, which are also non-haemolytic, have been considered as " typical food-poisoning " strains. Although such strains are most likely to survive cooking procedures, it is now well recognised that food poisoning can also be caused by strains that produce relatively heat-sensitive spores, and these strains may be haemolytic or non-haemolytic (Hauschild and Thatcher, 1967; Sutton and Hobbs, 1968; Hobbs, 1969). Food-poisoning strains of *C. welchii* are serotyped with two sets of antisera, one for the typical heat-resistant strains—Hobbs' types 1–24—and the other for the so-called heat-sensitive strains—types i–xviii (Hobbs *et al.*, 1973).

Collee was unable to show neuraminidase production in cultures of four British typical food-poisoning strains but, because culture supernates of two strains produced a myxovirus-receptor-inactivating effect on red cells (Collee, 1965*a*), he concluded that " more extensive investigation is required before it can be claimed that neuraminidase is never produced by typical food-poisoning strains " (Collee, 1965*b*).

Moss, Schekter and Cherry (1967), working in the United States, surveyed many strains of *C. welchii* for neuraminidase activity. These workers confirmed that the reference British heat-resistant food-poisoning strains examined by them did not produce neuraminidase, but they showed that one of five American food-poisoning strains of *C. welchii* that could not otherwise be differentiated from these strains was neuraminidase positive. American food-poisoning strains generally include many that are haemolytic or heat-sensitive or both; the majority of American food-poisoning strains examined by Moss *et al.* (1967) did produce neuraminidase.

In the present study, the more sensitive neuraminidase-assay procedure described by Fraser and Smith (1975) was used for a further investigation of neuraminidase production in a range of heat-resistant and heat-sensitive British food-poisoning strains of *C. welchii*.

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Materials and methods

Strains of C. welchii. Four classical haemolytic strains of *C. welchii* type A were used. Strain L2Ab was described in the previous paper (Fraser and Smith, 1975); strain L3A was also obtained from Professor C. L. Oakley, School of Medicine, University of Leeds. Strains C1 and 032 were laboratory strains originally isolated from wound infections in Edinburgh.

Typical non-haemolytic, heat-resistant, food-poisoning strains of *C. welchii* type A of Hobbs' types 1–4 (Nos. 8359, 8238, 8239 and 8247 respectively) were obtained from the National Collection of Type Cultures, Colindale, London; strains of Hobbs' types 5–24 were kindly provided by Dr Betty Hobbs, Central Public Health Laboratory, Colindale. Strains 029 and 153 were typical food-poisoning strains isolated from separate food-poisoning outbreaks in Edinburgh (Collee, 1965*b*); they were submitted to Dr Hobbs, who reported that they were both type-13 strains. Strains 611 and 4621 were provided by Dr Hobbs: these were non-haemolytic type-13 strains but were not responsible for actual food-poisoning outbreaks; strain 611 was isolated *post mortem* from a patient with colitis and strain 4621 from a patient who died during a food-poisoning outbreak attributed to a different strain of *C. welchii*. Reference food-poisoning strains of *C. welchii* type A that do not produce heat-resistant spores (types i–iii, v–xviii) were provided by Dr Hobbs, and are referred to as heat-sensitive strains.

Media and method of anaerobic culture. Cooked-meat broth and Proteose Peptone-water medium (PPW5) were described in the previous paper (Fraser and Smith, 1975). Todd-Hewitt Broth and Nutrient Broth (No. 2) were manufactured by Oxoid Ltd. Horse-blood agar contained Columbia Agar Base with 10% (v/v) defibrinated horse blood (Oxoid). Anaerobic culture methods were as previously described (Fraser and Smith, 1975) with a gas mixture of 10% (v/v) CO_2 and 90% H_2 used as a routine.

Observation of haemolytic effect. Strains were grown anaerobically on horse-blood agar for 18 h at 37°C with 10% CO_2. Strains graded as haemolytic produced clear haemolysis, with or without a surrounding zone of incomplete haemolysis. Non-haemolytic strains produced no effect or only a small zone of faint, incomplete haemolysis.

Serial culture of strains for tests of neuraminidase production. In general, a 0·1-ml inoculum of an overnight anaerobic cooked-meat broth culture was added to 10 ml of pre-steamed culture medium in a tube and incubated anaerobically for 48 h. Serial 48-h cultures were made in this manner with a 0·1-ml inoculum for each passage. A 5-ml sample of centrifuged supernate (800 *g* for 1 h at 4°C) from each passage was stored at −20°C. Cultures were incubated at 37°C unless otherwise indicated.

Disruption of washed cells. Five serial cultures were made in PPW5 broth, incubated on each occasion for 48 h at 39°C. Cultures 1, 2 and 3 were made by transferring 0·1 ml of culture into 10 ml of medium as for the serial cultures described above. For the fourth culture, a 1·0-ml inoculum was added to 100 ml medium and, for the fifth, 10 ml was added to 500 ml. Samples (5 ml) of centrifuged supernates (800 *g* for 1 h at 4°C) from the first four cultures were stored at −20°C. After centrifugation (2000 *g* for 1 h at 4°C) the supernate of the fifth serial culture was stored at −20°C and the packed cells were resuspended in 20 ml of sterile normal saline. The cells were washed three times in saline (2000 *g* for 1 h at 4°C) and the washed cells were resuspended in 20 ml of saline. The resuspended washed cells and samples of the supernate from each washing were held at −20°C.

The washed cell suspension was later thawed and four 3-ml samples of each organism were subjected to ultrasonic disruption (MSE-Mullard Ultrasonic Disintegrator) for 1 h with the samples held at 0°–1°C in an ice bath. This produced a great decrease in turbidity in the samples, and phase-contrast microscopy confirmed a reduction in the number of intact cells to less than 1% of the original. The treated samples were pooled and the centrifuged supernate (20,000 *g* for 1 h at 4°C) was held at −20°C.

Concentration and dialysis of culture supernates. Samples of culture supernate (100 ml) were concentrated in 2·5-cm Visking cellophane tubing exposed to polyethylene glycol (mol. wt. 6000) for 20 h at 4°C, and thereafter dialysed against large quantities of distilled water for 72 h at 4°C.

Assay for neuraminidase activity. The reagents and procedures are described in the previous paper (Fraser and Smith, 1975). The assay was standardised with reaction mixtures prepared as follows: test substance, 0·1 ml; sodium acetate buffer, *p*H 5·1, 0·15 ml; and substrate FVII diluted in the acetate buffer, 0·25 ml. The original batch of substrate, FVII(5), was used at a final concentration of 3·07 mg per ml in the 0·5-ml test volume and the second batch, FVII(6), at 4·0 mg per ml. Separate enzyme and substrate controls were assayed and used to correct the test results. Test and control mixtures were incubated at 37°C in a water bath for the appropriate length of time before the assay for free N-acetyl neuraminic acid (NANA). Duplicate mixtures were usually assayed, and the amount of NANA released is indicated in terms of the corrected average extinction values at 549 nm (E_{549}). In progress experiments, the timing was such that all tests, including time-zero (T_0) control tests, were assayed for NANA in one batch; T_0 test values were used for correction of test readings (Fraser and Smith, 1975).

Tests for the effects of culture materials on the NANA assay. These have been described in the previous paper, in which control assays and calculations are detailed (Fraser and Smith, 1975). Mixtures were prepared as for the standard neuraminidase assay but with 10 μg of pure NANA in 0·25 ml of distilled water in place of the substrate.

RESULTS

Production of neuraminidase by classical strains of C. welchii grown in various broth media

Four classical (haemolytic, heat-sensitive) strains of *C. welchii* type A were cultured for 48 h in a range of broth media, and a second serial culture was made from each in the same medium. The results of neuraminidase assays on the supernates of the second serial cultures (table I) indicate that neuraminidase production is poor in nutrient broth and generally good in PPW5 broth and Todd-Hewitt medium. These results reflect our experience with many strains of *C. welchii*; PPW5 broth and Todd-Hewitt medium give generally good yields of neuraminidase, but yields may vary markedly even in successive cultures of the same strain grown under apparently identical conditions in

TABLE I

Production of neuraminidase by four classical strains of Clostridium welchii type A grown in four broth media

Strain number	Result of neuraminidase assay on supernate from second serial culture in stated medium (E_{549})*			
	NB	CMB	THB	PPW5
L2Ab	0·315	0·732	1·320	0·640
L3A	0·056	0·190	0·656	1·052
C1	0·280	0·441	0·692	0·920
032	0·346	0·649	1·900	0·492

* The spectrophotometric value (E_{549}) is an average result obtained from duplicate 15-min. assay readings corrected by subtraction of the values for the substrate control and the corresponding enzyme control.

NB = nutrient broth; CMB = cooked-meat broth; THB = Todd-Hewitt broth; PPW5 = 5% Proteose Peptone water.

TABLE II

*Production of neuraminidase by heat-resistant and heat-sensitive reference food-poisoning strains of C. welchii type A**

Reference strains	Haemolytic effect on blood agar†	Serotype numbers of neuraminidase-positive strains‡	Serotype numbers of neuraminidase-negative strains§
Hobbs' types 1–24 (heat-resistant)	NH	13, 18, 21, 24	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 15, 16, 17, 19, 20, 22, 23
Hobbs' types i–xviii (heat-sensitive)	NH	i, ix	xiii, xiv, xv
	H	ii, iii, v, vi, vii, x, xi, xii, xvi, xvii, xviii	viii

* Tests performed on supernates of second serial 48-h cultures in PPW5 broth.
† See *Materials and Methods*; H = haemolytic; NH = non-haemolytic.
‡ Tests incubated with substrate FVII at 37°C. Supernates of most cultures gave clearly positive results in assays incubated for 15 min.; assays of supernates of strains belonging to serotypes 24 and i were incubated for 4 h to produce clearly significant assay values.
§ Tests incubated with substrate FVII at 37°C for 24 h gave negative results.

samples of medium prepared in a single batch (see fig. 1 below). On occasion, cultures in PPW5 broth were unduly mucoid; this occurred rather more frequently in Todd-Hewitt medium but was not correlated with variations in neuraminidase production. The amount of neuraminidase present in the supernate of cultures in PPW5 broth reaches a maximum within 24 h and there is no significant increase or decrease in activity between 24 and 72 h. In later studies we found that the production of neuraminidase by strain L2Ab in 48-h cultures in PPW5 broth was markedly influenced by the temperature of incubation of the culture within the range 35–42°C; the maximum

TABLE III

Production of neuraminidase by strain L2Ab and by five strains of C. welchii of Hobbs' type 13

Strain number	Result of neuraminidase assay on supernate from second serial culture in PPW5 broth (E_{549})*
L2Ab	0·579
NCTC10240†	0·149
029	0·163
153	0·172
611	0·531
4621	0·000‡

* See footnote to table I.
† Hobbs' type-13 reference strain.
‡ The negative result for the culture supernate of strain 4621 was confirmed when the period of incubation of the test was extended to 24 h.

TABLE IV

Neuraminidase activity in various culture products of strain L2Ab grown in PPW5 broth

Product of serial culture*	Result of neuraminidase assay (E_{549})†
Supernate 1	5·480
Supernate 2	2·120
Supernate 3	3·404
Supernate 4	3·672
Supernate 5	3·088
Supernate 5 concentrate‡	13·640
1st washing from bacillary deposit 5	0·433
2nd washing	0·032
3rd washing	0·021
Extract of sonicated washed deposit 5	1·428

* See *Materials and Methods*.
† See footnote to table I. (Samples diluted appropriately before assay).
‡ Concentrated to 22·1% of the original volume.

yield obtained at 39–40°C may be up to twice that obtained at 35°C. Minor differences in the temperature of incubation may contribute to the variation in amount of neuraminidase produced in serial cultures.

Production of neuraminidase by food-poisoning strains of C. welchii

We chose to test the supernate of the second serial 48-h culture in PPW5 broth at 37°C as a routine procedure in the present studies. The results of neuraminidase assays on 41 reference food-poisoning strains of *C. welchii* are presented in table II. The culture supernates were first tested in a screening assay incubated with FVII substrate for 1 h; thereafter, positive supernates were assayed at appropriate dilutions by a standard 15-min. assay procedure. Two supernates gave very low readings in the 1-h screening assay; these were assayed in tests incubated for 4 h to produce clearly significant readings. Supernates that were negative in the 1-h screening assay were then tested with 24-h incubation; all remained negative. Most of the typical non-haemolytic, heat-resistant *C. welchii* strains were neuraminidase negative, but there was good production of neuraminidase by the strains of Hobbs' types 13, 18 and 21 and the strain of Hobbs' type 24 produced a small amount of activity. Thirteen of the 17 heat-sensitive food-poisoning strains produced neuraminidase, but the remaining four strains were completely negative. It appears that in general non-haemolytic strains do not produce neuraminidase whereas haemolytic strains do, though there are clear exceptions to this general correlation.

A further four type-13 strains were tested for neuraminidase production in this way. Strains 029 and 153 were isolated from food-poisoning outbreaks in Edinburgh and strains 611 and 4621 were supplied by Dr Hobbs. The results presented in table III show production of a moderate amount of neuraminidase by strain 611 and rather small amounts by the reference strain 10240

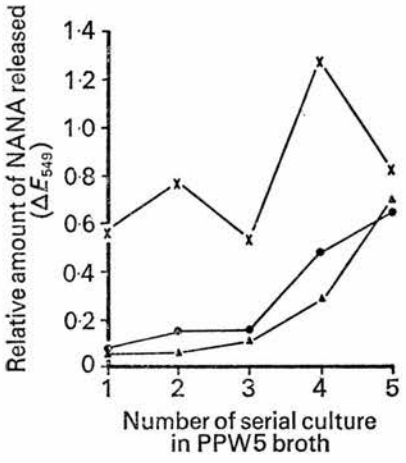

FIG. 1

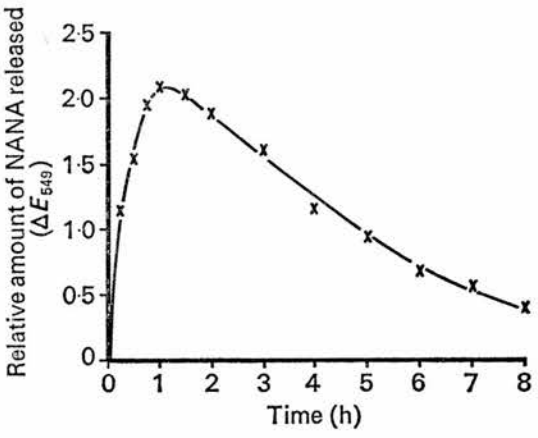

FIG. 2

FIG. 1.—Corrected test results of 15-min. neuraminidase assays of supernates of serial cultures of *C. welchii* strains in PPW5 broth: ×——×, strain L2Ab; ▲——▲, strain 153; ●——●, strain 029. Strain L2Ab supernates were assayed at a dilution of 1 in 4 in acetate buffer, *p*H 5·1, and the values corrected accordingly; supernates of strains 153 and 029 were assayed undiluted.

FIG. 2.—Release and destruction of NANA during neuraminidase assays on undiluted L2Ab cell extract for up to 8 h. The relative amount of free NANA is plotted as the spectrophotometric reading (E_{549}) of single-tube assays corrected by subtraction of the value for the time-zero test. The assay product for each test was diluted 1 in 5 in reagent 4 (acid butanol) before reading and the results were corrected accordingly.

and by strains 029 and 153; nevertheless, activity was clearly demonstrable after incubation of tests for 15 min. By contrast, there was no activity in the supernate of strain 4621 even when the tests were incubated for 24 h. When a sample of this supernate was incubated with NANA for 24 h under conditions equivalent to those of the neuraminidase assay there was no significant reduction in the assay value for NANA. Thus the negative result in the 24-h neuraminidase assay with the supernate of strain 4621 was not due to destruction of NANA by N-acyl neuraminic acid-aldolase (NAN-aldolase : N-acyl neuraminate lyase, EC 4.1.3.3).

Variability of neuraminidase production during serial culture

Five serial cultures of the classical strain L2Ab and the weakly neuraminidase-positive food-poisoning strains 029 and 153 were made in 10-ml volumes of PPW5 broth. The results of the standard 15-min. assay for neuraminidase activity in the supernates of these cultures are presented in fig. 1. Strain L2Ab produced moderate but variable yields of neuraminidase in all supernates. Initially, the two Edinburgh food-poisoning strains produced small amounts of neuraminidase but this increased in serial cultures until by the fifth culture the activity was comparable with that produced by strain L2Ab. The presence of neuraminidase activity in the supernates of the

first cultures in this series was confirmed by demonstrating that more NANA was released when the period of incubation was extended. In tests in which samples of supernate were incubated with a standard amount of NANA for 15 min., there was no significant breakdown of NANA. The values plotted in fig. 1 therefore give a valid indication of the relative amounts of neuraminidase produced by the three strains in this experiment. These results might suggest that sequential culture in PPW5 broth of strains 029 and 153 produces a gradually increased yield of neuraminidase. However, further experiments with organisms derived from freeze-dried stocks and grown under apparently identical conditions did not show this pattern; the initial cultures often produced high amounts of the enzyme and variations in yield could not be attributed to any particular variable in experimental conditions.

Assays for cell-associated neuraminidase

Five serial cultures of the neuraminidase-positive classical strain L2Ab and the neuraminidase-negative food-poisoning strain Hobbs' 2 were made successively at 39°C in 10-, 10-, 10-, 100- and 500-ml volumes of PPW5 broth. Samples (100 ml) of culture supernates from the fifth serial cultures were concentrated approximately five-fold; the L2Ab culture was concentrated to 22·1% of its original volume, and the Hobbs' type-2 culture to 17·8%, after dialysis (see *Materials and Methods*). The washed cells from the 500 ml of the fifth serial cultures were resuspended in 20 ml saline and disrupted ultrasonically; the resultant suspension was then centrifuged free of cell debris and is referred to as cell extract. Table IV presents the results of neuraminidase assays on the culture products of strain L2Ab from this experiment. The 15-min. neuraminidase assays show very high yields in the supernates of all

TABLE V

Effect of incubating N-acetyl neuraminic acid (NANA) with culture supernates of strain L2Ab and the Hobbs' type-2 strain for up to 24 h

Reaction mixture*	Result of assay for NANA (E_{549})† after incubation of mixture for stated time (h)					
	0	½	2	3	22	24
NANA alone	0·514	0·514	0·506	0·530	0·531	0·537
NANA+supernate of 5th serial culture of strain L2Ab	0·516	0·502	0·474	0·474	0·299	...
NANA+supernate of 5th serial culture of Hobbs' type-2 strain	0·503	0·500	0·494	0·511	0·511	0·500
NANA+concentrated supernate of 5th serial culture of Hobbs' type-2 strain	0·548	0·524	0·523	0·523	0·493	0·476

* A standard amount of NANA, 10 μg in 0·5-ml final volume, was tested.

† Spectrophotometric reading (E_{549}) of single-tube assays corrected by subtraction of the value for the corresponding time-zero enzyme control.

... = Not done.

five serial cultures and concentration of a sample of the fifth culture supernate produced a proportional increase in activity. The amount of neuraminidase in the successive washings fell rapidly to trace amounts, but there was release of a significant amount of neuraminidase after ultrasonic treatment of the suspension of L2Ab cells. By contrast, neuraminidase activity was not detected in any of the equivalent samples from the cultures of strain Hobbs' 2, although the tests were incubated for 24 h.

Table V shows the amount of NANA that was destroyed during incubation with samples of the fifth culture supernates for periods up to 24 h under conditions equivalent to those of the neuraminidase assay. The supernate of the fifth culture of strain L2Ab produced slight destruction of NANA after incubation for 24 h; this suggests that there is slight NAN-aldolase activity in the culture supernate (although too little to interfere significantly with the 15-min. neuraminidase assay). There was, however, no destruction of NANA by the supernate of the fifth culture of the strain of Hobbs' type 2 and even with this product concentrated five-fold there was only a very slight reduction in the assay value for NANA after incubation for 24 h. This confirms that the results of neuraminidase assays on culture supernates of the strain of Hobbs' type 2 are valid, i.e. that this strain produced no demonstrable extracellular neuraminidase.

Disruption of the washed cells of strain L2Ab and the strain of Hobbs' type 2 liberated NAN-aldolase into the supernate. The results presented in table VI show that in each case the supernate from the third washing of the cells in saline produced no destruction of NANA but that the supernate after disruption of the cells was able to destroy the test dose of NANA during incubation for 24 h. The amount of NAN-aldolase activity in the cell extracts of the two strains appears to be comparable, as judged by the similar rate of NANA destruction during the first 3 h of the test.

TABLE VI

Effect of incubating NANA with cell extracts of strain L2Ab and the Hobbs' type-2 strain for up to 24 h

Reaction mixture*	Result of assay for NANA (E_{549})† after incubation of mixture for stated time (h)					
	0	½	1	2	3	24
NANA+3rd washing of strain L2Ab	0·477	0·467	0·481	0·474	0·470	0·461
NANA+cell extract of strain L2Ab	0·460	0·334	0·250	0·125	0·086	0·020
NANA+3rd washing of Hobbs' type-2 strain	0·485	0·502	0·494	0·487	0·489	0·492
NANA+cell extract of Hobbs' type-2 strain	0·480	0·372	0·300	0·163	0·113	0·008

* A standard amount of NANA, 10μg in 0·5-ml final volume, was tested.

† Spectrophotometric reading (E_{549}) of single-tube assays corrected by subtraction of the value for the corresponding time-zero enzyme control.

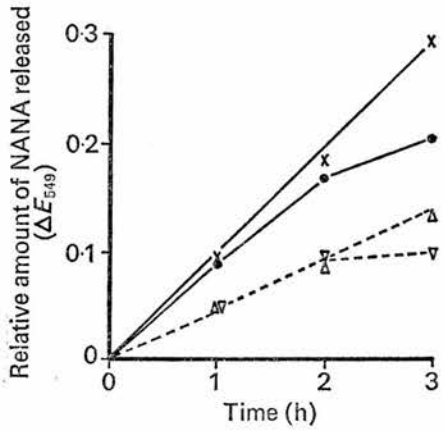

FIG. 3.—Results of neuraminidase assays incubated for periods up to 3 h in the presence of cell extract of the Hobbs' type-2 strain. The test mixtures (final volume 0·6 ml) contained: 0·25 ml FVII substrate; 0·15 ml sodium-acetate buffer, *p*H 5·1; 0·1 ml L2Ab cell extract diluted in the acetate buffer; and either 0·1 ml acetate buffer or 0·1 ml undiluted cell extract from the Hobbs' type-2 strain.

2% (v/v) L2Ab cell extract+buffer, ×——×; 2% L2Ab cell extract+cell extract from the Hobbs' type-2 strain, ●——●; 1% L2Ab cell extract+buffer, △——△; 1% L2Ab cell extract+cell extract from the Hobbs' type-2 strain, ▽——▽. Results corrected by subtraction of the value for the corresponding time-zero test.

Fig. 2 shows the effect of the NAN-aldolase in the L2Ab cell extract when the neuraminidase assay is done at intervals up to 8 h. The amount of NANA released increases rapidly to reach a peak at 1 h and thereafter there is a progressive fall in assay value; thus the result of the 15-min. neuraminidase assay on the L2Ab cell extract given in table IV is liable to be an underestimate. Because the cell extract prepared from the Hobbs' type-2 strain contains a similar amount of NAN-aldolase activity, the negative result in the 24-h neuraminidase assay on this sample might have been due to subsequent destruction of the NANA released; however, further studies showed no detectable release of NANA even during the first 3 h of incubation of assay mixtures containing cell extract of this strain.

To give an indication of the amount of neuraminidase that we could expect to detect in the Hobbs' type-2 cell extract despite its NANA-destroying activity, dilutions of L2Ab cell extract were assayed in the presence and absence of undiluted cell extract. The results are presented in fig. 3. The assay values for neuraminidase were reduced by the Hobbs'-2 cell extract in tests incubated for 2-3 h, but even 1% of the amount of neuraminidase released by ultrasonic treatment of strain-L2Ab cells could clearly be detected in the presence of the cell extract prepared from the Hobbs' type-2 strain.

DISCUSSION

Neuraminidase-positive strains of *C. welchii* type A produce small amounts of neuraminidase when grown in nutrient broth but large amounts, and

sometimes very large amounts, in the PPW5 broth that we chose for these studies. We found considerable variation in the amount of neuraminidase produced by strain L2Ab in serial cultures incubated under apparently identical conditions, even when the PPW5 broth was prepared in a single batch and when we assayed the supernates of the serial cultures in a single experiment.

Collee (1965*b*) found that four non-haemolytic, heat-resistant food-poisoning strains were neuraminidase negative and Moss *et al.* (1967) confirmed that 12 reference British food-poisoning strains (Hobbs' types 1–3 and 5–13) were neuraminidase negative. It seemed that it might be generally true that non-haemolytic, heat-resistant food-poisoning strains did not produce neuraminidase, but Moss *et al.* (1967) went on to demonstrate an exception with one American isolate that was otherwise indistinguishable from the Hobbs' reference strains. Most of the American food-poisoning isolates that these workers examined differed in one or more characters from the non-haemolytic, heat-resistant Hobbs' strains, and the majority of these American strains did produce neuraminidase. We have now studied 41 of the current British reference food-poisoning strains and found that, whereas 12 of 17 so-called heat-sensitive strains were neuraminidase positive, 20 of 24 heat-resistant strains were neuraminidase negative. Our findings show that, in British as in American food-poisoning strains, the ability to cause food-poisoning is not correlated with lack of neuraminidase production, although the majority of non-haemolytic heat-resistant isolates are neuraminidase negative.

The four heat-resistant strains that we found to be clearly neuraminidase positive include the reference strain of Hobbs' type 13, which was found to be neuraminidase negative by Moss *et al.* (1967). We found that three other type-13 strains produced neuraminidase, including strains 029 and 153, which were amongst the strains reported as neuraminidase negative by Collee (1965*b*). It is possible that our strains might have altered as a result of prolonged storage and subculture, although their characteristics were carefully re-checked to confirm that they were still non-haemolytic and produced heat-resistant spores. PPW5 medium is generally much better for the production of neuraminidase than the media used by Collee in 1965 and it is unlikely that the less sensitive assay procedure then in use, with egg-white as substrate, would have detected the small amounts of neuraminidase produced in some cultures of these strains even in PPW5 broth—for example, in the experiment illustrated in fig. 1. Although the data from this experiment suggest that sequential subculture in PPW5 broth might result in a gradually increased yield of the enzyme, this pattern was not found in subsequent experiments. We have found considerable variation in the amount of neuraminidase produced by strains 029 and 153 in PPW5 broth, but all cultures that we have examined contain neuraminidase activity that is clearly demonstrable by our present methods. We think that our ability to show neuraminidase production by strains 029 and 153 is now probably attributable to developments in our culture and assay procedures; however, even after incubation of tests for 24 h, we found no evidence of neuraminidase production by one other type-13 strain. It thus appears that there is true variation in the ability to produce neuraminidase among strains of a single Hobbs' serotype of *C. welchii*.

Our finding that any neuraminidase-positive strain may vary considerably in its production of the enzyme despite apparently identical conditions of culture led us to assay the supernates of five sequential cultures in tests of an apparently neuraminidase-negative Hobbs' type-2 strain. When strain L2Ab was grown in PPW5 broth at the optimum temperature for neuraminidase production it produced very high yields of the enzyme, which was essentially extracellular; some further neuraminidase could be released by ultrasonic disintegration of the washed cells. When the Hobbs' type-2 strain was grown in parallel under identical conditions,

no activity was demonstrable in culture supernates even after five-fold concentration, and no neuraminidase was released by ultrasonic disintegration of the cells.

As our assay depends upon the detection of NANA liberated by the neuraminidase, the presence of NAN-aldolase might produce a false negative result. However, there was insignificant NAN-aldolase activity in the culture supernate of the Hobbs' type-2 strain; this confirms the validity of our 24-h neuraminidase assay as a sensitive test for neuraminidase activity and demonstrates that the Hobbs' type-2 strain produces no extracellular neuraminidase (i.e. less than 0·01% of the neuraminidase produced by strain L2Ab). When the neuraminidase-positive L2Ab cells were disrupted, the NAN-aldolase released produced some interference with the neuraminidase assay. A similar amount of NAN-aldolase was released from the neuraminidase-negative Hobbs' type-2 strain, but our further experiments showed that this would not prevent the demonstration of quite small amounts of neuraminidase—as little as 1% of that associated with L2Ab cells. Thus we now have clear evidence that British food-poisoning strains of *C. welchii* include some that are neuraminidase positive and some that still cannot be shown to produce neuraminidase despite considerable advances in our ability to detect the enzyme.

Whilst it is unlikely that neuraminidase is concerned in the mechanism of pathogenicity of *C. welchii* in the gut, it is possible that the enzyme plays a part in gas gangrene. Hauschild and Thatcher (1968) found that whereas non-haemolytic, heat-resistant food-poisoning strains did not produce experimental gas gangrene in animals, and that this was correlated with production of very low amounts of the lethal α-toxin, some non-haemolytic but heat-sensitive food-poisoning strains did produce gas gangrene if injected in large numbers. There was a clear but imperfect correlation between virulence and in-vitro production of α-toxin. Although the mechanism of pathogenicity of *C. welchii* in wound infection has long been associated with the organism's ability to produce α-toxin (Evans, 1945; MacLennan, 1962), Bullen (1970) has developed an interesting argument to suggest that other factors may be more important. It might be argued that the neuraminidase, which is produced in close association with the α-toxin by many strains, plays a hitherto undetected role as an aggressin (Collee, 1965*b*) and that anti-neuraminidase in *C. welchii* antitoxin (see Warren and Spearing, 1963) may contribute to its protective effect. Under these circumstances, the organism's infectivity and virulence might depend upon its early production of neuraminidase *in vivo*. Our ability to define neuraminidase-negative strains might now be exploited to test this suggestion.

Summary

The production of neuraminidase by a classical strain of *Clostridium welchii* (*C. perfringens*) type A was studied. Good yields were produced in 5% Proteose Peptone-water medium (PPW5); the enzyme was essentially extracellular but some further neuraminidase could be released by ultrasonic disintegration of the cells. This also released N-acyl neuraminic acid-aldolase (NAN-aldolase) and the degree to which this interferes with the assay for neuraminidase was evaluated.

Forty-one British reference food-poisoning strains of *C. welchii* type A were examined for extracellular neuraminidase production in PPW5. Twelve

of 17 strains that produce so-called heat-sensitive spores were neuraminidase positive whereas 20 of 24 strains that are non-haemolytic and produce very heat-resistant spores were neuraminidase negative. Variation was found in the ability to produce neuraminidase among strains of a single Hobbs' serotype; four Hobbs' type-13 strains produced neuraminidase but a fifth did not. Disruption of the cells of a Hobbs' type-2 strain that did not produce any extracellular neuraminidase released NAN-aldolase but there was no evidence of cell-associated neuraminidase. British food-poisoning strains of *C. welchii* type A thus include some that are clearly neuraminidase positive and some that still cannot be shown to produce neuraminidase. There is no correlation between lack of neuraminidase production and the ability to cause food poisoning, although the majority of non-haemolytic heat-resistant strains do not produce neuraminidase. It remains possible that neuraminidase may play a part in *C. welchii* gas gangrene; it is suggested that the ability to define neuraminidase-negative strains may now be of value in investigating this possibility.

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NEURAMINIDASE PRODUCTION BY CLOSTRIDIA

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THE assay for neuraminidase (EC 3.2.1.18) developed by Fraser and Smith (1975) was used by Fraser and Collee (1975) to investigate the production of the enzyme by a variety of strains of *Clostridium perfringens* (*C. welchii*) type A. Sensitive assay procedures were developed that allowed definition of truly neuraminidase-negative strains. Many classical haemolytic strains of *C. perfringens* type A produced neuraminidase and most non-haemolytic food-poisoning strains did not.

It has been suggested that neuraminidase might play a part in the production of gas gangrene by *C. perfringens* and other clostridia (Collee, 1965*b*; Gadalla and Collee, 1968; Müller, 1974). Our previous studies of *C. perfringens* have now been extended to investigate the production of neuraminidase by a range of other pathogenic and non-pathogenic clostridia.

MATERIALS AND METHODS

Bacterial strains. The strains used and their sources are detailed in tables I–III. All strains were held as lyophilised stock, with periodic subculture in cooked-meat broth (CMB) and re-lyophilisation. The source of strains that were recently obtained from the National Collection of Type Cultures, Colindale, London, is shown as NCTC. Other strains were originally obtained from the NCTC and have been held for many years in the collection of Professor J. G. Collee in this department; their NCTC numbers are quoted. The remainder of the strains, including some NCTC strains and clinical isolates from a variety of infections, were obtained from various sources and have been held here, sometimes for many years and with many subcultures. Donors of these strains included Dr Nancy Hayward, Monash University Medical School, Australia, the late Professor C. L. Oakley, Leeds University Medical School, Dr B. Watt, Central Microbiological Laboratories, Western General Hospital, Edinburgh, and colleagues in the diagnostic laboratories in this Department.

For the present studies, all strains were freshly grown in CMB from lyophilised stock and their purity and identity were carefully rechecked in cultural and biochemical studies. Some strains were further checked by immunofluorescence microscopy.

Media and method of anaerobic culture. CMB medium and proteose peptone water broth (PPW5) were prepared as described by Fraser and Smith (1975). The PPW5 broth was clarified by filtering through Whatman no. 1 paper before autoclaving. To improve the growth of strains of *C. novyi*, L-cysteine (Koch Light) and dithiothreitol (BDH) were added (Moore, 1968); the supplemented broth is referred to as PPW5S. A freshly-prepared concentrated ($\times 50$) solution containing cysteine and dithiothreitol was passed through a membrane filter (0·2-μm pore) and added aseptically to the sterile PPW5 broth to give final concentrations of the two substances of 1 mg per ml and 90 μg per ml respectively; the PPW5S was presteamed and cooled to 37°C, just before inoculation with the test organism.

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Todd Hewitt broth (THB) was prepared according to the manufacturer's recommendations (Oxoid). In early experiments the *p*H was adjusted to 7·4 before autoclaving but it was found that the *p*H of THB sometimes rose considerably on storage. In later experiments the *p*H was adjusted after autoclaving, by the addition of membrane-filtered 1·0M HCl to the sterile broth before use.

Anaerobic culture methods were as previously described (Fraser and Smith, 1975). Cultures were prepared from lyophilised stock and maintained in CMB medium. An inoculum (0·1 ml) of an overnight anaerobic culture in CMB incubated at 37°C was added to 10 ml presteamed medium and incubated anaerobically at 37°C for 48 h. A second serial 48-h culture was made with an inoculum of 0·1 ml from the first 48-h culture. The purity of growth in the second serial culture was carefully checked. When an organism did not grow or grew very poorly in a particular medium, a further attempt was made with an inoculum of 1·0 ml, incubating each serial culture for 96 h.

Growth was estimated visually and recorded as follows: +++, dense turbidity; ++, moderate turbidity; +, light turbidity or faint turbidity with many cells seen by microscopy in a wet film; ±, faint turbidity with few cells present on microscopy; −, no turbidity, or no cells seen on microscopy.

The supernates of the second serial cultures were tested for neuraminidase activity. The cultures were centrifuged (800 *g* for 60 min. at 4°C) and 5-ml samples of the centrifuged supernates were stored at −20°C before assay. In addition, the centrifuged supernates of *C. botulinum* cultures were passed through a membrane filter (0·2-μm pore) to ensure the removal of all organisms.

Preparation of cell extracts. Cell extracts were prepared from the centrifuged cell deposits of the second serial cultures of a number of strains grown in PPW5 or CMB medium. Cooked-meat particles were allowed to settle and the broth was removed for centrifugation. All the supernate was removed from the packed cell deposit after centrifugation (1200 *g* for 90 min. at 4°C) and the cells were resuspended in 2 ml sterile normal saline. The resuspended cells were subjected to ultrasonic disintegration for 15–30 min. in an ice bath until the sample was no longer turbid (Fraser and Collee, 1975). These uncentrifuged cell extracts were stored at −20°C until assayed for neuraminidase.

Substrate for neuraminidase assays. Two batches of human glycoprotein substrate FVII (Fraser and Smith, 1975) were used. Batch FVII(6) was used at a final concentration in the 0·5-ml test volume of 4·0 mg FVII per ml as previously (Fraser and Collee, 1975); this batch contained about 4·2% of *N*-acetyl neuraminic acid (NANA) and the final concentration of releasable NANA in the reaction mixture was thus *c.* 168 μg per ml. The concentration of NANA-rich glycoprotein may vary in different batches of FVII and it is preferable to express the concentration of substrate used in assays in terms of the amount of NANA that can be released from the substrate. Batch FVII(7) was used at a final concentration of 140 μg NANA per ml.

Assay for neuraminidase activity. The standard assay was as described by Fraser and Collee (1975). Initial assays for neuraminidase activity in culture products were incubated for 60 min. before assay for free NANA. Test spectrophotometric readings at 549 nm were corrected by subtraction of the sum of the values for separate enzyme and substrate controls. When the 60-min. assay clearly showed neuraminidase activity, the assay was repeated with a 15-min. incubation period. When the 60-min. assay showed little or no activity, tests were incubated for 24 h in order to demonstrate the presence or absence of detectable neuraminidase activity.

Tests that gave low corrected spectrophotometric readings (E_{549}) were further examined with a Pye-Unicam SP8000A spectrophotometer to determine whether the absorption peak was at 549 nm. In order to demonstrate clearly whether a low assay value was or was not due to the presence of NANA, it was occasionally necessary to read the absorption curve of the test mixture against a time-zero (T_0) control test (Fraser and Smith, 1975), rather than the enzyme control, as reference sample.

The corrected results of neuraminidase assays have been graded as follows: +++, clearly detectable in 15-min. assays ($E_{549}>0{\cdot}1$); ++, low spectrophotometric values in

15-min. assays (<0·1) but NANA release clearly demonstrable in 60-min. assays; +, not demonstrable in 60-min. assays but high values (>0·5) in 24-h assays; ±, low values (<0·5) in 24-h assays but absorption peak clearly shown at 549 nm; −, no peak at 549 nm demonstrable in 24-h assays.

Tests for the effects of culture products on the NANA assay. Tests to demonstrate whether false negative results might be attributable to destruction of NANA by *N*-acetyl neuraminic acid-aldolase (NAN-aldolase EC 4.1.3.3) were performed on all culture supernates or cell extracts that gave low positive (+ or ±) or negative results in the 24-h assay for neuraminidase (see Fraser and Smith, 1975). Culture products that did not reduce the assay value for 10 μg NANA (E_{549} *c.* 0·5) by 0·1 or more during incubation for 24 h were regarded as having insignificant NANA-destroying activity.

Effect of Ca^{2+} and EDTA on neuraminidase assays. $CaCl_2 . 2H_2O$ (BDH, Analar) or EDTA (ethylenediaminetetra-acetic acid disodium salt, BDH, Analar) were added to the acetate buffer used in the neuraminidase assay. The final concentrations of added Ca^{2+} or EDTA in the reaction mixtures were 3mM Ca^{2+}, 1mM Ca^{2+}, 1mM EDTA or 5mM EDTA. The enzyme preparation (0·1 ml) and the acetate buffer containing Ca^{2+} or EDTA (0·15 ml) were mixed and held at 37°C for 30 min. before addition of the substrate FVII (0·25 ml) and the start of incubation of the neuraminidase assay. Separate enzyme and substrate control tubes contained the equivalent volumes of acetate buffer in place of the substrate and enzyme respectively. Enzyme control assays also contained the appropriate amount of added Ca^{2+} or EDTA.

The *C. perfringens* neuraminidase tested was a dialysed preparation (P9D3) of supernate from a bulk culture of strain L2Ab in PPW5 broth (P9) prepared as for the standard preparation (P9D2) used by Fraser and Smith (1975). *Vibrio cholerae* Receptor-Destroying Enzyme (Wellcome; RDE) was used as *V. cholerae* neuraminidase.

RESULTS

Neuraminidase production by Clostridium perfringens, Clostridium septicum, Clostridium chauvoei and Clostridium tertium

Table I gives the data for production of neuraminidase by various strains and species of clostridia examined in this study. Strain L2Ab, a classical neuraminidase-producing strain of *C. perfringens* type A, grew well in both PPW5 and THB media, producing large amounts of neuraminidase in the culture supernates. Cell-associated neuraminidase was also demonstrable with the PPW5 culture, but the enzyme is predominantly extracellular. Good production of neuraminidase was also found in PPW5 culture supernates of strains of *C. perfringens* types B, C, D and E.

The production of large amounts of neuraminidase by strains of *C. septicum* is also easily shown by these techniques and the results for strain NCTC547 are typical of those obtained with other strains of *C. septicum.*

The two strains of *C. chauvoei* tested did not grow well in PPW5 broth and they failed to grow in THB medium. Nevertheleless, strain CC2 produced moderate amounts of neuraminidase in the PPW5 culture supernate. Neuraminidase production by strain NCTC8070 was demonstrated when the incubation time of the assay was prolonged to 24 h; the enzyme was predominantly extracellular. The low values for neuraminidase activity in the culture supernate and the cell extract were not due to NAN-aldolase activity.

Two strains of *C. tertium* produced small amounts of neuraminidase; more enzyme was produced in PPW5 culture supernates although the organisms grew better in THB medium.

TABLE I

Production of neuraminidase by Clostridium perfringens, C. septicum, C. chauvoei, C. tertium, C. sordellii and C. bifermentans in two broth media

Test organism	Growth in stated medium*		Relative amount of neuraminidase activity† in culture product			Source of organism
			PPW5		THB	
	PPW5	THB	Culture supernate	Cell extract	Culture supernate	
C. perfringens						
L2Ab	+++	+++	+++	+++	+++	*See* Fraser and Smith (1975)
C. septicum						
NCTC547	++	+	+++	+++	+++	*See* Gadalla and Collee (1967)
C. chauvoei						
CC2	+‡	−‡	++	...	...	Collee
NCTC8070	+‡	−‡	+	±	...	NCTC
C. tertium						
CTl	+	++	+	±	±	Watt
NCTC541	+	++	+	...	±	NCTC
C. sordellii						
1734	+++	+	+++	...	+++	Oakley
P3	+++	+	+++	...	+++	Oakley
CB2	+++	+	+++	...	+++	Collee
CB3	++	++	+	±	+	Collee
CB4	++	++	+	±	+++	Watt
NCTC1340	++	+	++	...	+++	NCTC
NCTC2914	+	+	+	...	++	NCTC
NCTC6800	+	++	+	...	+	NCTC
NCTC6801	+++	+	++	...	++	NCTC
NCTC6927	++	++	+	...	++	NCTC
NCTC6929	++	++	++	...	++	NCTC
NCTC8780	+++	+	+++	...	+++	NCTC
C. bifermentans						
B4	+++	+++	−	−	−	Oakley
1617	+++	+++	−	−	−	Oakley
NCTC506	+++	++	−	−	−	Collee
NCTC1341	++	++	−	...	−	NCTC
NCTC6928	++	+++	−	...	−	NCTC

* See *Methods* for assessment of growth. PPW5 = Proteose peptone water; THB = Todd Hewitt broth.

† Culture supernate or cell extract from second serial culture in stated medium was tested. See *Methods* for grading of neuraminidase activity; ... = not tested.

‡ 96-h culture; 1 ml inoculum.

Studies with Clostridium sordellii and Clostridium bifermentans

Most of the 12 strains of *C. sordellii* grew well in PPW5 broth and the supernates of all these cultures contained neuraminidase (table I). These cultures in PPW5 broth gave high enzyme-control readings (E_{549}) due to the formation of a chromogen with a peak absorption at 530 nm (Fraser and Smith, 1975) and it was occasionally necessary to use a time-zero (T_0) control test as

reference sample (see *Methods*). The enzyme was predominantly extracellular in the two strains tested for intracellular enzyme. Several strains grew rather poorly in THB medium but neuraminidase activity was still clearly demonstrable in the culture supernates. Enzyme control values (E_{549}) were low in THB culture supernates and there was no difficulty in demonstrating the absorption peak at 549 nm of the chromogen formed in neuraminidase assays.

By contrast, the five strains of the closely related *C. bifermentans* examined could not be shown to produce neuraminidase activity in culture supernates of either medium. The values for enzyme control tests were high in assays with PPW5 culture supernates but comparison with T_0 control tests confirmed that there was no increase in value in tests incubated for 24 h. No cell-associated enzyme activity was shown in 24-h assays with the cell extracts of PPW5 cultures of three strains. These negative results were shown not to be due to presence of NAN-aldolase activity.

Studies with Clostridium novyi (oedematiens)

No neuraminidase was demonstrable in cultures of 10 strains of *C. novyi*, types A to D. Table II shows that some strains grew poorly in the test media, although the PPW5 broth was supplemented with cysteine and dithiothreitol (PPW5S), and a large inoculum and prolonged incubation time were used. No

TABLE II

Growth of Clostridium novyi obtained in three fluid media

Test organism	Growth in stated medium*		
	PPW5S	THB	CMB
Type A			
GR2A	+	++	...
GR4A	+	++	...
NCTC538 (GR1A)	+	++	+
NCTC6737 (GR3A)	+	++	...
Type B			
GR1B	±†	+†	+
Type C			
NCTC9746‡	+†	+†	...
NCTC9747 (GR1C)	+†	+†	++
Type D			
NCTC8145 (GR2D)	+	++	...
NCTC8350 (GR1D)	++	++	++
NCTC9692‡	++	++	...

* See *Methods* for assessment of growth; ... = not tested. PPW5S = Proteose peptone water supplemented with cysteine and dithiothreitol; THB = Todd Hewitt broth; CMB = cooked-meat broth.

† 96-h culture; 1 ml inoculum.

‡ Source, NCTC. For the source of all organisms, except those marked ‡, see Rutter and Collee (1969).

neuraminidase activity could be shown in culture supernates in PPW5S or THB media even in assays incubated for 24 h. Culture supernates and cell extracts of four strains grown in CMB medium were also neuraminidase-negative. No significant NANA destruction was found with the majority of these culture supernates or cell extracts; the remainder produced a small reduction in the assay value for NANA after incubation for 24 h, but in no instance was this fall in reading (E_{549}) greater than 0·15 and this would not have obscured significant neuraminidase activity.

TABLE III

Growth of other neuraminidase-negative clostridia obtained in two broth media

Test organism	Growth in stated medium*		Source of organism
	PPW5	THB	
C. tetani			
NCTC279	++	++	NCTC
NCTC540	+++	+	Collee
NCTC5404	++	++	Collee
NCTC5405	++	+	NCTC
NCTC5413	++	+	Collee
NCTC9569	±†	+	NCTC
C. botulinum			
Type A, NCTC7272	+++	++	NCTC
Type B, NCTC7273	+++	++	NCTC
Type C, NCTC3732	−†	+++	NCTC
Type E, NCTC8266	+	++	NCTC
C. sporogenes			
23	+++	+++	Hayward
24	+++	+++	Hayward
26	+++	+++	Hayward
28	+++	+++	Hayward
C. histolyticum			
CH2	+++	+++	Collee
NCTC503	+++	++	Collee
NCTC7123	+++	+++	Collee
NCTC7124	+++	+++	Collee
C. butyricum			
NCTC7423	±†	++	Collee
C. sphenoides			
NCTC507	+	+++	NCTC
C. fallax			
NCTC8380	+++	++	NCTC
C. tetanomorphum			
NCTC2909	++	+++	NCTC
C. subterminale			
CS1	++	+++	Watt

* See *Methods* for assessment of growth. PPW5 = Proteose peptone water; THB = Todd Hewitt broth.

† 96-h culture; 1 ml inoculum.

Studies with other neuraminidase-negative clostridia

Table III shows the other strains of clostridia that did not produce neuraminidase; no activity was demonstrable in 24-h assays in any of the culture supernates. A few strains that grew poorly in one of the test media showed better growth in the other. None of the culture supernates contained significant NANA-destroying activity.

Effect of Ca^{2+} and EDTA on neuraminidase assays

Table IV compares the effect of varying the concentration of Ca^{2+} in the assays of neuraminidase from *C. perfringens* and *V. cholerae*. Assays of pure NANA were not affected by the presence of these amounts of Ca^{2+} or EDTA. The *V. cholerae* enzyme is very calcium-dependent and its activity in our assay can be almost doubled by the addition of 3mM Ca^{2+} to the reaction mixture. By contrast, the activity of the *C. perfringens* enzyme is not increased by the addition of 3mM Ca^{2+}; 5mM EDTA reduced the assay value by *c.* 21% only.

The calcium dependence of the neuraminidase produced by other clostridia was assessed under our standard assay conditions with no added Ca^{2+} and with added 3mM Ca^{2+}. Neuraminidase-containing PPW5 culture supernates of the following strains were tested (see table I): *C. perfringens* L2Ab; *C. septicum* NCTC547; *C. chauvoei* CC2 and NCTC8070; *C. tertium* CT1 and NCTC541; *C. sordellii* CB3 and NCTC8780. Culture supernates with strong neuraminidase activity were diluted in the acetate buffer before testing in 15-min. assays and the time of incubation of assays of supernates with weak neuraminidase activity was prolonged in order to give assay results in the range 0·1–0·6. In no instance was there increased activity in the presence of the added Ca^{2+}.

TABLE IV

Effect of Ca^{2+} and EDTA on the assay of neuraminidase from Clostridium perfringens and Vibrio cholerae

Concentration of added Ca^{2+} or EDTA in reaction mixture*	Result of neuraminidase assay on stated preparation (E_{549})†	
	C. perfringens (P9D3)	*V. cholerae* (RDE)
3mM Ca^{2+}	0·348	0·576
1mM Ca^{2+}	0·315	0·436
None added	0·351	0·318
1mM EDTA	0·313	0·202
5mM EDTA	0·277	0·012

* See *Methods*.

† The spectrophotometric value (E_{549}) is an average result obtained from duplicate 15-min. assay readings corrected by subtraction of the values for the corresponding substrate and enzyme controls. For tested enzyme preparations P9D3 and RDE, see *Methods*.

Further tests were performed with the PPW5 and THB culture supernates of *C. bifermentans* strains B4, NCTC506, NCTC1341 and NCTC6928 that could not be shown to contain neuraminidase in standard assays (see table I). The 24-h assays remained negative in the presence of added 3mM Ca^{2+}.

Discussion

The neuraminidase of *C. perfringens* type A was one of the first to be discovered (McCrea, 1947) and has been well characterised (Cassidy, Jourdian and Roseman, 1966). Collee (1965*a*) reported that the enzyme was also produced by strains of types B, C, D and E and this was confirmed during the present studies. Although most classical strains of *C. perfringens* type A produce large amounts of neuraminidase, some produce only small amounts and a number of food-poisoning strains are neuraminidase-negative (Fraser and Collee, 1975).

An organism that is shown to produce the enzyme is clearly neuraminidase-positive; it is more difficult to be certain that an organism is truly neuraminidase-negative and can never produce it. The assay procedures developed by Fraser and Collee (1975) to define true neuraminidase-negative strains of *C. perfringens* were used in the present study. Neuraminidase-positive clostridia were shown to produce the enzyme in culture supernates in both PPW5 and THB media although there were often considerable differences in the relative amounts produced and these did not correlate clearly with differences in the relative amounts of growth in the two media. Neuraminidase-negative strains could not be shown to produce the enzyme in either medium, even when assays were incubated for 24 h. Clostridial neuraminidase is essentially extracellular but several neuraminidase-negative strains were further tested to confirm that no cell-associated enzyme could be detected after disruption of the cells.

The *p*H optimum of a particular neuraminidase may vary considerably when tested with different substrates (Rafelson, Schneir and Wilson, 1963), with different buffer ions (Cassidy *et al.*, 1966; Fraser and Smith, 1975) or at different ionic strengths (Rosenberg and Schengrund, 1976). Optimal *p*H values quoted for bacterial neuraminidases are usually in the range 4·5–6·5 (Drzeniek, 1972); in the present studies, acetate buffer at *p*H 5·1 was used as this was found to be optimal for *C. perfringens* neuraminidase in earlier studies with FVII substrate (Fraser and Smith, 1975).

Neuraminidases from different sources vary in their calcium dependence and their sensitivity to EDTA (Boschman and Jacobs, 1965; Drzeniek, 1972). *C. perfringens* neuraminidase is not calcium-dependent (Cassidy *et al.*, 1966) and the present studies showed that the enzyme produced by other clostridial species is also calcium-independent. Ca^{2+} was not added to our assays as a routine, but culture products of *C. bifermentans* and *C. novyi* retested with the addition of Ca^{2+} remained negative. It is thus unlikely that our inability to demonstrate neuraminidase production by other clostridia was due to lack of Ca^{2+} in the reaction mixtures.

The assay for *C. perfringens* neuraminidase may be complicated by the

presence of NAN-aldolase which may destroy the NANA that is released, but this does not usually prevent detection of quite small amounts of neuraminidase in our assay (Fraser and Collee, 1975). In the present studies, none of the culture products assayed contained sufficient NANA-destroying activity to interfere significantly with the assays for neuraminidase; negative results are thus not attributable to destruction of released NANA by NAN-aldolase.

The clostridial species that produced neuraminidase in the present study were *C. perfringens*, *C. septicum*, *C. chauvoei*, *C. tertium* and *C. sordellii*; all strains tested were neuraminidase-positive. The other clostridial species tested were all neuraminidase-negative.

C. septicum and *C. chauvoei* are usually regarded as separate species but the differences between them are very small; Willis (1969) discussed the arguments for regarding them as two types of a single species. Warren and Spearing (1963) noted the presence of neuraminidase activity in a lyophilised preparation of *C. septicum* toxin. Gadalla and Collee (1968) found that the enzyme was produced by all of 15 strains studied. Müller and Schallehn (1972) detected the enzyme in two strains of *C. chauvoei* (*C. feseri*) by electrophoretic methods. The present study confirmed that strains of *C. septicum* generally produce large amounts of neuraminidase whereas the two strains of *C. chauvoei* produced rather small amounts under the same test conditions.

Howe *et al.* (1957) reported that *C. tertium* produced an enzyme that destroyed myxovirus receptor substances. Müller and Werner (1974) demonstrated neuraminidase production by a strain of *C. tertium* and the present study confirmed that small amounts of the enzyme were produced by two other strains.

C. sordellii and *C. bifermentans* are very closely related and there has been considerable debate as to whether or not they should be regarded as separate species (see Willis, 1969). Pathogenic strains of *C. sordellii* produce the β-toxin whereas *C. bifermentans* is non-toxigenic, but this is not an adequate criterion for classification as non-toxigenic strains of *C. sordellii* also occur. Nakamura *et al.* (1975) used numerical taxonomy and DNA homology in a reinvestigation of this problem. They scored for 162 features (not including neuraminidase activity) and concluded that the two species could be reliably differentiated by only a few biochemical tests—fermentation of mannose and sorbitol, urease activity, arginine deamination and growth inhibition by mannose. Warren and Spearing (1963) noted neuraminidase activity in a preparation of *C. sordellii* toxin, and White and Mellanby (1969) separated the neuraminidase from the toxic activity in the culture supernate of a strain of *C. sordellii*. The present finding that all of 12 strains of *C. sordellii* produced neuraminidase but that none of five strains of *C. bifermentans* did so suggests that this may be another character that distinguishes the two species. Soloviev *et al.* (1972) have suggested that tests for neuraminidase production may be of value in studies of vibrio taxonomy and it now appears that they may also be of interest in certain aspects of clostridial taxonomy.

C. sordellii is a rare cause of wound infection in man and animals (Willis, 1969). The finding that neuraminidase production is one of the few features

that distinguish this organism from the non-pathogenic *C. bifermentans* might suggest that neuraminidase plays a part in the pathogenicity of *C. sordellii*. White and Mellanby (1969) were unable to show an enhancing effect when *C. sordellii* neuraminidase was added to β-toxin in guinea-pig skin tests, but they did not study actual infection with the organism.

It has been suggested that neuraminidase may play a part in the pathogenesis of clostridial infections (Collee, 1965*b*; Gadalla and Collee, 1968; Müller, 1970*a* and *b*; Müller and Schallehn, 1972; Fraser and Collee, 1975) although there is little direct evidence for this. Neuraminidase action has been shown to alter a wide range of properties and functions of important proteins and cells (tabulated by Rosenberg and Schengrund, 1976). Müller (1974) reviewed the possible role of neuraminidase in bacterial infections. He argued that there is a correlation between virulence and the ability to produce large amounts of neuraminidase *in vivo* for a variety of strains and species of bacteria and he suggested that neuraminidase-producing organisms are more likely to be successful in a wide range of infections.

Gadalla and Collee (1968) showed that neuraminidase is produced *in vivo* during experimental infections with *C. septicum*, and similar findings have been made in tissues taken from guinea-pigs with experimental *C. perfringens* gas gangrene (unpublished results). Müller (1970*b*) demonstrated changes attributable to neuraminidase in the glycoproteins of the wound exudate from a patient with a *C. septicum* infection and this also shows that neuraminidase is produced and active *in vivo*.

Neuraminidase might conceivably influence an organisms's invasiveness, or its ability to gain access to a target site or receptor. *C. septicum* and classical strains of *C. perfringens* type A both produce large amounts of neuraminidase and are important causes of gas gangrene in man, in whom they are invasive pathogens when infection is established. However, *C. novyi* type A, the other important cause of gas gangrene, is also invasive and is neuraminidase-negative. Of the other neuraminidase-positive species, *C. chauvoei* is pathogenic for animals (blackquarter) but not for man and *C. tertium* is regarded as non-pathogenic. *C. tetani* and *C. botulinum*, both neuraminidase-negative, are important non-invasive and essentially toxic human pathogens. *C. sporogenes*, which is closely related to *C. botulinum*, is neuraminidase-negative and essentially non-pathogenic, but *C. histolyticum* and *C. fallax* are neuraminidase-negative histotoxic clostridia with invasive potential. The other clostridia found to be neuraminidase-negative in this study are regarded as non-pathogenic (see Willis, 1969 and 1977).

It is clear that there is no strict correlation between neuraminidase production and the ability of various clostridial species to cause wound infection. However, several pathogenic species produce neuraminidase in addition to recognised toxins and it is possible that this may contribute to their virulence. Further studies are in progress to determine whether there is any correlation between virulence and neuraminidase production in strains of those species that produce the enzyme.

SUMMARY

The production of neuraminidase (EC 3.2.1.18) by a range of clostridial species was investigated with techniques previously developed to distinguish neuraminidase-negative and neuraminidase-positive strains of *Clostridium perfringens* (*welchii*). Large amounts of extracellular neuraminidase were produced by representative strains of *C. perfringens* and *C. septicum* in the test media. Under similar conditions, two strains each of *C. chauvoei* and *C. tertium* were found to produce small amounts of the enzyme. All of 12 strains of *C. sordellii* were clearly shown to produce neuraminidase, often in large amounts, but none of five strains of the closely related but non-pathogenic *C. bifermentans* had demonstrable neuraminidase activity. No neuraminidase was produced by *C. novyi* (*oedematiens*) types A-D (10 strains), *C. tetani* (6), *C. botulinum* types A, B, C or E (4), *C. sporogenes* (4), *C. histolyticum* (4) or by single strains of five other clostridial species.

Clostridial neuraminidase was predominantly extracellular and was not calcium-dependent. The investigation took account of variations in growth and enzyme production in different media. It was necessary to prolong the neuraminidase-assay reaction time to 24 h and to monitor for the presence of NAN-aldolase (EC 4.1.3.3) to define true negatives. It is suggested that neuraminidase production may be of value in taxonomic studies and that its production by several pathogenic species of clostridia may be of interest in studies of pathogenicity and virulence.

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FOOD POISONING CAUSED BY CLOSTRIDIUM PERFRINGENS (C. WELCHII) TYPE A

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Although *Clostridium perfringens (C. perfringens)* type A occurs regularly as a commensal in the gut of man and animals, some strains are able to cause food poisoning in man if ingested in large numbers. An association between diarrhoea and *C. perfringens* was first suggested in 1895 but despite sporadic reports thereafter the difficulty of discriminating food-poisoning strains from commensal organisms in the faeces prevented the general recognition of the disease[1].

The work of Hobbs and her colleagues[2] in Britain finally established *C. perfringens* as a definite cause of food poisoning. These studies identified "typical food-poisoning strains" that grow as non-haemolytic colonies on horse-blood agar and produce unusually heat-resistant spores. Such organisms have been classified as type-A_2, to distinguish them from so-called classical strains of type-A_1[3]. Although type-A_2 strains are the more likely to survive cooking procedures, because of the marked heat resistance of their spores, later studies in Britain and America established that food poisoning can also be caused by classical type-A_1 strains that produce relatively heat-sensitive spores[4,5,6].

The term "typical food-poisoning strain" has probably outlived its usefulness. It is now clear that the food-poisoning potential of a strain of *C. perfringens* rests upon its ability to produce a specific enterotoxin during sporulation in the gut (see below). Enterotoxin may be produced by either haemolytic or non-haemolytic strains. Classical strains are haemolytic on horse-blood agar because of the production of theta (θ) toxin. Strains that do not produce θ toxin are defined as non-haemolytic-though they may produce a weak haemolytic effect due to the action of their alpha (α) toxin. The spores produced by classical type-A_1 strains of *C. perfringens* are not very heat-resistant and are usually inactivated by boiling at 100°C within a few minutes, whereas type-A_2 food-poisoning strains form markedly heat-resistant spores can survive boiling in cooked-meat broth or food for several hours[2]. Markedly heat-resistant spores are typically produced by strains that are virtually non-haemolytic, but not all non-haemolytic strains produce heat-resistant spores.

Techniques for detecting enterotoxin produced during sporulation in laboratory media have only recently been developed and few epidemiological studies have differentiated between strains of *C. perfringens* on this basis. However, the recognition of the enterotoxin is a major step forward in our understanding of the mechanism of pathogenicity of this disease and raises the possibility of improved methods for its diagnosis.

The occurrence of *Clostridium perfringens*

C. perfringens occurs very widely in nature. The spores are able to survive under adverse conditions and can be recovered from soil and dust, water, mud and grass. The ecology of this organism has not been fully studied. *C. perfringens* type A occurs regularly in the faecal flora of man and animals and can be found in human faeces in numbers that range from relatively few to about 10^4 to 10^5 per gram wet weight of faeces[7]. Although the organism does not readily sporulate in routine culture media, it does so in the human gut and considerable numbers of spores of *C. perfringens* type A_1 may occur in faeces. Sutton[8,9] studied the carrier rate of type-A_2 strains

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and related high carrier rates (15-25%) to communal feeding and to poor hygiene.

Many opportunities arise for spores of *C. perfringens* to contaminate meat and meat products at the abattoir, in transit to shops and market places, and in catering establishments and in the home. As the organism is encountered so frequently and so widely, it is not usual to attempt to trace the ultimate source in an outbreak of food poisoning but rather to determine the circumstances or conditions that allowed the almost inevitable contamination to be boosted during processing of the food concerned.

Growth of *C. perfringens* in food

It can be argued that the organism has taken advantage of changing habits in food production because bulk food processing and communal eating, particularly of meat foods, produces the conditions necessary to deliver high challenge doses of *C. perfringens* to man. The bulk cooking of meat allows circumstances in which heat gain is slow and subsequent cooling may be prolonged. Dissolved oxygen is driven off and the meat maintains an anaerobic environment. Spores of *C. perfringens* contaminating the meat may be activated and germinate readily under these conditions[10]. They they multiply very rapidly at temperatures up to 45°C. A portion of such food may taste normal but will contain many hundreds of millions of viable *C. perfringens* organisms which pass the acid barrier of the stomach in such a meal and sporulate in the human gut, producing the enterotoxin.

The heat-resistance of type-A_2 spores allows them to survive the whole cooking procedure, whereas type-A_1 spores are not likely to cause trouble unless they are introduced by faulty catering practice during the period between cooking and serving.

C. perfringens food poisoning and its incidence

The signs and symptoms of *C. perfringens* food poisoning include abdominal cramping pain and diarrhoea, usually beginning within about 9-14 hours after ingestion of the infected food and persisting for a day or so. The patient is usually not fevered and vomiting is not characteristic. The effective challenge dose is about 10^8 - 10^9 viable organisms; the numbers of organisms in the faeces of those involved in an outbreak range from 10^5 10^8 per gram with many spores present. In an outbreak, only a proportion of those at risk report that they have been significantly unwell and it seems that there is a considerable variation in host susceptibility.

Pre-cooked meat and poultry foods are most commonly involved in this form of food poisoning, but fish dishes, milk foods and leguminous seeds are sometimes associated with outbreaks. The numbers of cases of *C. perfringens* food poisoning reported in England and Wales in recent years are disturbingly high (see Table). *C. perfringens* was the second most common cause of food poisoning, being responsible for approximately 25% of general outbreaks in 1973-5[12]. As the condition is associated with bulk-cooked foods, the numbers of people affected in an outbreak are usually large - often 50-300, and up to 13,500 persons have been affected in a single episode[13]. Salmonellae are the commonest cause of food poisoning in Britain, but salmonella outbreaks tend to affect smaller numbers of people and there are many more limited family outbreaks and sporadic cases.

It is disappointing that the numbers of cases of *C. perfringens* food poisoning continue to be so high, because the occurrence of *C. perfringens* food poisoning is a direct indictment of the food-processing procedures of the institution involved and the condition is clearly preventable by observing simple fundamental rules of hygiene in food processing[14].

The prevalence of *C. perfringens* food poisoning in many countries of the world is not known, because notification is either inadequate or non-existent, but there are many indications in the literature that *C. perfringens* food poisoning is under-reported even in those countries in which the condition is well recognised and where there are laboratory facilities for its detection[13].

(C.perfringens Enterotoxin)

Our understanding of the role of enterotoxin in *C. perfringens* food poisoning stems largely from the systematic studies of two groups, Duncan and colleagues in Wisconsin, and Hauschild and colleagues in Ottawa[15]. These workers established valid animal models for the study of *C. perfringens* food poisoning, similar to those developed over the previous decade to demonstrate the central role of *Vibrio cholerae* enterotoxin in the pathogenesis of cholera[16]. Hauschild, Niilo & Dorward[17,18] worked with lambs and showed that, when suitably administered, human food-poisoning strains caused experimental diarrhoea and produced fluid accumulation in ligated intestinal loops. Independently, Duncan and colleagues produced similar results with infant rabbits[19,20]. Living organisms were used in the initial ligated loop experiments, but a cell-free enterotoxin was soon demonstrated by Duncan & Strong[21] in studies with rabbits and by Hauschild, Niilo & Dorward[22,23] in lambs. The enterotoxin could be specifically neutralised by experimental antisera and was quite distinct from the α toxin and other toxins of *C. perfringens* type A.

A good correlation was found in these and other studies between the production of enterotoxin able to cause fluid accumulation in ligated loops of rabbit or lamb intestine and the ability to cause expermental food poisoning when suitably administered to man or monkeys[24, 25] Enterotoxin-negative strains did not produce experimental food poisoning.

Enterotoxin production and sporulation

The production of food-poisoning symptoms in human volunteers requires the ingestion of large numbers of vegetative cells of a food-poisoning strain of *C. perfringens* type A[26, 5, 24]. Culture filtrates or heat-killed cutures of the organism are seldom effective. The reason for this became clear when it was found that enterotoxin production is linked to sporulation. It is unique in this, as other clostridial toxins are produced as exotoxins during vegetative growth. Enterotoxin is not found in the medium until the cells lyse although it can be released by sonic disintegration during the early stages of sporulation[21, 22, 23, 27]. Strains of *C. perfringens* type A do not generally spore readily in the laboratory, although spores are commonly formed in the gut and are regularly present in faeces. A few unusual strains produce many spores in laboratory media, but this is quite uncommon. The introduction of Duncan and Strong's improved sporulation medium (DS medium) has proved a great advance as many strains of *C. perfringens* sporulate well in it[28, 29, 30] Sporulation is essential for enterotoxin production; it should be borne in mind that not all strains that sporulate readily are enterotoxin producers.

C. perfringens in poisoned food is present predominantly as actively growing vegetative cells and these release the enterotoxin when they sporulate in the intestine. The amount of free enterotoxin present in food or in culture filtrates of *C. perfringens* is usually inadequate to cause symptoms when taken orally, Hauschild, Walcroft & Campbell[31] produced vomiting and diarrhoea in monkeys dosed with purified enterotoxin, and Skjelkvale & Uemura in 1977 produced diarrhoea in human volunteers by giving very large oral doses (8-12 μg) of *C. perfringens enterotoxin*[32]

Enterotoxin as a spore-specific product

Duncan, Strong & Sebald's studies with mutants of *C. perfringens* showed that enterotoxin is a sporulation-specific gene product but its precise role in sporulation remains obscure[33]. Later studies confirmed that enterotoxin production is an early event (c. stage IV) in sporulation[34, 35] and suggested that the enterotoxin is also one of the normal struetural proteins of the spore coat[36, 37]. It may be that food - poisoning strains over-produce this spore-coat protein, which accumulates in the cytoplasm of the sporulating cells; enterotoxin production my be the result of poor control of this stage in sporulation[36]. During sporulation, some enterotoxin-producing strains form a paracrystalline inclusion which may represent an aggregation of enterotoxin in the cytoplasm of sporulating cells[38]. Niilo used direct microscopy with fluorescent antibody to confirm the production of enterotoxin in the cytoplasm of sporulating cells but did not demonstrate antibody on the surface of washed spores[39], perhaps because it lies in the deeper layers of the spore coats.

Properties of enterotoxin and methods of assay

The enterotoxin is a simple heat-labile acidic protein of M.W. *c.* 36,000 which has been purified and characterised by various techniques[40, 41, 42, 43, 44, 45].

In addition to its local action on the gut, it also has a lethal effect if injected intravenously into mice, guinea pigs, rabbits or lambs, but only when large doses are given[46, 47]. When injected into the skin of rabbits or guinea pigs, it produces an erythematous reaction[48, 49] and this provides a more convenient assay than mouse lethality or the use of ligated intestinal loops[50, 47, 51].

More recently, immunological techniques have been developed for sensitive *in-vitro* assays[52]. Duncan & Somers[53] introduced electroimmunodiffusion, and Uemura, Sakaguchi & Riemann[27] developed a reversed passive haemagglutination (RPHA) assay. Naik & Duncan found the RPHA technique rather variable although it is very sensitive, detecting as little as 0.001 μg enterotoxin per ml; these workers recommended a counterimmunoelectrophoresis assay for rapid detection and quantitation of enterotoxin (limit 0.2 μg per ml)[54].

Relationship to other bacterial enterotoxins

The *C. perfringens* type A enterotoxin is clearly distinct from the enterotoxins produced by *Vibrio cholerae*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus*, Salmonellae and shigellae[55]. However, Skjelkvale & Duncan[56, 57] studied other types of *C. perfringens* and found that type-C strains also produce an enterotoxin which is biochemically and immunologically indistinguishable from that of the type-A strains; this raises the question whether the enterotoxin may play a part in the pathogenesis of *C. perfringens* type C necrotic enteritis.

Pathogenesis of *C. perfringens* food poisoning

Since the disease is very rarely fatal, little is known of the response of the human gut during the illness. In experimental animals the small bowel is most sensitive to the effect of enterotoxin[58] and it is presumed that this is also the site of in man. There is, however, little direct information on the site of replication, sporulation and enterotoxin production by *C. perfringens* in the human gut.

It is known that in a variety of gut infections adhesive factors play an important part in assisting colonisation of the intestinal mucosa at an appropriate level in the gut, e.g. K88 and K99 antigens in *Escherichia coli* enteritis in pigs and cattle, and similar factors may be important in cholera[59]. Arbuckle noted that type-

C strains of *C. perfringens* which cause a form of enteritis in piglets can attach to the intestinal epithelium of the area of the gut that they affect[60]. No adhesive factor or antigen has been identified for enteropathogenic strains of *C. perfringens* type A so far.

It is clear that the enterotoxin is the factor responsible for producing fluid accumulation in ligated segments of small intestine in experimental animals. This is a local effect and it is not thought that there is significant absorption of enterotoxin during the clinical illness. Large doses have to be given parenterally in order to produce systemic effects[46]. Circulating antibody to enterotoxin is present in many healthy people [61], but it appears to give no protection against further attacks in man or experimental animals[26, 24, 62, 32].

There are clear differences in the mode of action of the enterotoxins of *C. perfringens* and *V. cholerae*. The enterotoxin of *V.cholerae* does not damage the intestinal epithelium, but binds to it and stimulates active secretion of fluid and electrolytes into the gut lumen, with cyclic AMP as the mediator of this effect [16]. Like *V. cholerae*, *C. perfringens* does not invade the intestinal epithelium, but the *C. perfringens* enterotoxin does cause some damage to the epithelium at the tips of the ileal villi in rabbits. The biochemical changes suggest a general pattern of cell degeneration and metabolic inhibition and cyclic AMP has not been found to be involved in the clostridial model[63, 64, 65].

Diagnosis of *C. perfringens* food poisoning

Careful bacteriological investigations are required to confirm the clinical and epidemiological impression that an outbreak of food poisoning is due to *C. perfringens* type A. Detailed recommendations for laboratory procedures are given by Hobbs[66], Hobbs *et al*[67], Hauschild[13] and Willis[68]. Hauschild *et al*[69]. have compared various media for the enumeration of *C. perfringens* in foods and a further study of media for the examination of faeces is in progress.

Samples of suspect foods should be examined if possible but they are often not available by the time the diagnosis is suggested. The organism will be present as vegetative cells in food and the samples should not be heated before culture. Faecal specimens from affected people should be examined for spores of *C. perfringens*. If faecal samples are boiled before culture, heat-resistant strains will be isolated but the spores of many enterotoxin-producing strains will be killed by this treatment. Mild heating is, however, of value in recovery of heat-sensitive *C. perfringens* from faeces as heat shock activates the spores and increases the rate of germination.

The simple demonstration of *C. perfringens* in the faeces of affected people is of limited value. The qualitative evidence requires to be substantiated by quantitative evidence, because many foods are contaminated with *C. perfringens* and the organism occurs regularly in the faeces of normal people. It should be appreciated that the count of organisms in remnants of suspect food will depend on the conditions under which the food has been kept and little reliance can be put upon high counts unless the food was held in the refrigerator after serving. Under normal circumstances, median counts of the organism do not usually exceed 10^5 per gram of food or faeces, whereas in *C. perfringens* food poisoning the affected food may contain very high counts of *C. perfringens* (up to 10^9 per gram) and the faeces of affected people may contain *C. perfringens* in counts of 10^5 - 10^8 per gram for some days after the food-poisoning incident. These counts fall within a few weeks so that a temporary convalescent carrier state persists. Sutton[9] was able to distinguish between recent cases and carriers of food-poisoning strains of *C.perfringens* in this way.

When significant numbers of *C. perfringens* have been found in the faeces of a number of affected people, these should be compared for haemolytic activity and heat resistance in order to show whether they appear similar to each other and to any isolate from the suspect food. The identity of the strains can then be confirmed by serotyping in a reference laboratory. Several isolates from each sample should be sent as more than one serotype may be found.

Serotyping of *C. perfringens*

Various attempts have been made to develop serotyping methods for *C. perfringens*[70]. Two sets of antisera were developed to identify type-A_2 or type-A_1 food-poisoning strains[2,67], but there was considerable overlap between the two groups and recent studies have aimed at producing a unified set of antisera for all *C. perfringens* type A strains, regardless of haemolytic activity or the heat resistance of the spores[71]. It is hoped soon to produce an internationally standardised scheme.

There does not appear to be a constant relationship between specific serotypes and enterotoxin production, but the demonstration that substantial numbers of the same serotype are present in a suspect food and the faeces of affected people is the best evidence to incriminate *C. perfringens* type A in an outbreak of food poisoning.

Lecithinase-negative strains

Clostridium perfringens type A is identified primarily by its production of toxins (see Willis[70, 68]), notably the lethal α-toxin, a lecithinase or phospholipase C which is not involved in the pathogenesis of food poisoning. Strains of *C. perfringens* type A vary in the amount of α-toxin produced, but type-A_2 strains are usually only weakly toxigenic[2]. It is difficult to identify strains of *C. perfringens* that do not produce lecithinase, because this is the cornerstone of our identification system; but such lecithinase-negative strains do occur[72] and it has recently been shown that such strains may occasionally be responsible for food poisoning [73].

Further approaches

It has only recently become practicable to test isolates directly for enterotoxin production and it seems desirable that this should now be used to supplement the standard epidemiological and serological techniques. Uemura, Sakaguchi & Riemann[27] found increased *in-vitro* production of enterotoxin after strains had been subjected to heat treatment, and this was confirmed by Skjelkvale & Duncan [56] with type-A and type-C strains. The precise mechanism of this enhancement is uncertain but Uemura[74] recommended that heat treatment should be performed on three successive cultures of each isolate before testing for enterotoxin production.

The significance of the detection of enterotoxin-positive strains in the faeces of an individual is not yet clear. Most strains isolated from outbreaks of food poisoning produce enterotoxin in DS medium, while few studies have shown enterotoxin-positive strains in healthy human subjects or the natural environment[75]. The ecology of these strains has not been properly studied and the ultimate source of food-poisoning strains has seldom been traced. It is not known whether enterotoxin-producing strains are particularly liable to contaminate and grow in food, or whether the conditions found in bulk-cooked meat dishes select or induce enterotoxin production. However, using triple heat treatment, Uemura was able to demonstrate enterotoxin-production by strains of *C. perfringens* type A from 11 of 35 (31%) healthy human subjects[74]. This observation underlines the continuing importance of quantitative epidemiological studies.

Recent studies in India have shown a higher incidence of enterotoxin-positive

strains of *C. perfringens* in faeces of patients with diarrhoea than in non-diarrhoeal controls[76, 77] but there is little information on the incidence or epidemiology of *C. perfringens* food poisoning in India.

The development of very sensitive immunological assays such as RPHA also raises the possibility of making the diagnosis by detecting enterotoxin directly in faeces. This approach is recommended by Skjelkvale & Uemura[32] who were able to detect amounts of 0.2-16 μg enterotoxin per gram of faeces after oral administration of purified enterotoxin to volunteers.

Conclusion

C.perfringens type A has been increasingly recognised as a common cause of outbreaks of food poisoning in Britain and America during the past 25 years. Its incidence in developing countries is not known, but it seems likely to become commoner wherever there is a move towards centralised catering in canteens and institutions. Typical outbreaks affect fairly large numbers of people who have eaten cold or reheated meat dishes that have been cooked in bulk but not held under proper conditions before serving.

The recognition over the last 10 years that the symptoms are produced by an enterotoxin has greatly advanced our understanding of the pathogenesis of the disease although the ecology of enterotoxin-producing strains remains unclear. *C. perfringens* type A is a normal commensal in the gut of man and of animals and its spores are widely distributed in nature. Meat and other foods are almost inevitably contaminated with the organism, which can multiply rapidly if bulk-cooked food is not adequately cooled and held refrigerated before serving. Prevention depends upon careful attention to fundamental rules of good catering practice.

The diagnosis of *C. perfringens* type A food poisoning is complicated by the need to distinguish food-poisoning from commensal strains in the faeces. Strains that produce particularly heat-resistant spores are most likely to survive cooking and cause trouble, but outbreaks may also be due to enterotoxin-producing strains that form relatively heat-sensitive spores. Careful epidemiological studies must be followed by prompt qualitative and quantitative bacteriological examination of the faeces of affected people and, where possible, of suspect foods also. The development of sensitive methods for detection of enterotoxin production during sporulation in the laboratory should lead to further improvements in our procedures for recognition of the condition.

TABLE

Numbers of cases of *C. perfringens* type A food poisoning in England and Wales. 1969-75*

Years	Number of cases
1969	1534
1970	1263
1971	978
1972	1026
1973	1311
1974	2769
1975	2418

*from Vernon & Tillett [78] and Vernon[12]

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NEURAMINIDASE PRODUCTION BY BACTEROIDACEAE

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SUMMARY. The production of neuraminidase (EC 3.2.1.18) by 77 strains of Bacteroidaceae was investigated by techniques previously used to study neuraminidase production by clostridia. Conditions for culture and assay of *Bacteroides fragilis* neuraminidase were characterised. The enzyme is predominantly cell associated; it is not calcium dependent and the *p*H optimum for its production is *c.* 4·5.

Most neuraminidase-positive *Bacteroides* strains produced the enzyme well in the test media but a few strains failed to produce it consistently in one or other of the media. Because of these occasional variations, strains were grown and tested in at least two media before being defined as neuraminidase negative.

Within the *B. fragilis* group of species, *B. fragilis*, *B. vulgatus*, *B. distasonis*, *B. ovatus*, *B. thetaiotaomicron* and *B. variabilis* were neuraminidase positive while *B. eggerthii*, *B. uniformis* and *B. splanchnicus* were negative. Two subspecies of *B. melaninogenicus* (ss. *melaninogenicus* and ss. *levii*) were positive but the other (ss. *intermedius*) was negative. Strains of *B. oralis* and *B. bivius* produced the enzyme while *B. ruminicola*, *B. disiens*, *B. asaccharolyticus* and *B. corrodens* did not. The microaerophilic *B. ochraceus* were also positive. None of the *Fusobacterium* or *Leptotrichia* species tested produced neuraminidase.

Our results for neuraminidase production are consistent for all strains of each species examined and we suggest that tests for neuraminidase production would be a valuable addition to biochemical tests currently used in taxonomic studies of the Bacteroidaceae.

INTRODUCTION

The importance of the *Bacteroides-Fusobacterium* group of gram-negative anaerobic bacilli as significant pathogens in various infections has become increasingly apparent with recent improvements in methods for their isolation and identification (Finegold, 1977). Little is known of their mechanisms of pathogenicity, and the biochemical attributes of the different species are still being defined.

Neuraminidases (EC 3.2.1.18) are produced by many species of aerobic and

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anaerobic bacteria (see Müller, 1974). The enzyme is thought to play a significant role in infection with influenza virus and other myxoviruses (Drzeniek, 1972; Rosenberg and Schengrund, 1976) and it has been suggested that it may also be a virulence factor for some pathogenic bacteria (Collee, 1965; Müller, 1974).

Müller and Werner (1970*a* and *b*) reported production of neuraminidase by some *Bacteroides* species and argued that the enzyme had a role in pathogenesis. We have previously reported studies of production of neuraminidase by clostridia (Fraser and Collee, 1975; Fraser, 1978) and have now extended these studies to a range of well characterised strains representing the major species of gram-negative non-sporing anaerobic bacilli.

Materials and methods

Bacterial strains. Many of the strains were characterised in detail in this laboratory and described by Duerden, Holbrook, Collee and Watt (1976) or Deacon, Duerden and Holbrook (1978). The other strains studied were as follows: *Bacteroides eggerthii* NCTC11155, *B. splanchnicus* NCTC10825 and NCTC10826, *B. asaccharolyticus* NCTC9337 and *Fusobacterium varium* NCTC10560; these were obtained from the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT. *B. uniformis* VPI11227, *B. variabilis* VPI11368 and *B. melaninogenicus* ss. *levii* VPI3300 were obtained from Dr Lillian V. Holdeman, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24060, USA. *B. oralis* VPI8906D and VPI9958, *B. bivius* VPI5540, VPI6318, VPI6822 and VPI7880, and *B. disiens* VPI7852 and VPI8057 were provided by Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Colney Lane, Norwich NOR 7OF. *B. ruminicola* strains B38024, B38080 and B56029 were provided by Dr T. Mitsuoka, Animal Pathology Laboratory, Institute of Physical and Chemical Research, Wako, Saitama 351, Japan. *B. corrodens* strains 143A and 151RV were provided by Dr A.L. James, Department of Chemistry, Faculty of Science and Technology, Newcastle upon Tyne Polytechnic. The GNAB and WPH strains were isolated from various sites in healthy adults in this laboratory, or from clinical material by colleagues in the diagnostic laboratories in the Bacteriology Departments of the Royal Infirmary and the Western General Hospital, Edinburgh. All strains were identified to species level by the methods described by Duerden *et al.* (1976).

Culture media and method of anaerobic culture. Cooked-meat broth (CMB), 5% proteose peptone water broth (PPW5) and Todd–Hewitt broth (THB) were prepared as described by Fraser (1978). Thioglycollate broth (TGB) was prepared according to the manufacturer's recommendations (Thioglycollate Medium, fluid, without dextrose or Eh indicator, code 11727; BBL, Becton Dickinson UK Ltd, York House, Empire Way, Wembley, Middlesex HA9 0PS) with the addition of 0·25% yeast extract (Oxoid) and 0·25% sodium succinate (BDH) to improve growth of *Bacteroides* strains. Digest broth (DB) was prepared as described by Cruickshank *et al.* (1975) but with best beef steak in place of horse flesh; the $NaHCO_3$ was omitted and the final *p*H adjusted with NaOH to 7·4–7·6 before sterilisation by autoclaving. PPY broth medium was as described by Deacon *et al.* (1978). The BM broth medium used in this study was prepared as described by Deacon *et al.* (1978) but 0·25% sodium succinate was added and horse serum omitted.

CMB was used without added growth factors but all other media were supplemented with haemin and menadione as a routine; in addition, cysteine hydrochloride and Na_2CO_3 were added to PPY and BM media. Solutions of these supplements were sterilised by filtration and added aseptically to the steamed media just before inoculation as described by Deacon *et al.* (1978). The final concentrations were: haemin, 5 μg/ml; menadione, 1 μg/ml; cysteine hydrochloride, 0·075% (w/v); and Na_2CO_3, 0·04% (w/v).

Anaerobic culture methods were those described by Fraser and Smith (1975). Cultures were prepared from lyophilised stock and maintained in CMB. For standard tests, an inoculum of 0·2 ml of a 48–h anaerobic culture in CMB incubated at 37°C was added to 10 ml of steamed medium and incubated anaerobically at 37°C for 48 h. Growth was estimated visually and recorded as $+++$, $++$, $+$, $\pm$ or $-$ as described by Fraser (1978). Incubation was continued to 96 h to improve growth when there was $<+$ turbidity at 48 h.

Preparation of cell extracts. The 48–h or 96–h cultures were centrifuged (800 *g* for 60 min at 4°C). If the culture supernates were to be tested, 5-ml samples of the centrifuged supernates were removed and stored at −20°C. The rest of the supernate was then removed from the packed cell deposit and the cells from the 10 ml culture were resuspended in 2 ml of sterile normal saline. The resuspended cells were subjected to ultrasonic disintegration for 15–20 min in an ice bath (Fraser and Collee, 1975) and these uncentrifuged cell extracts were stored at −20°C until assayed for neuraminidase.

A more concentrated bulk cell extract was prepared for tests of the effects of *p*H and Ca^{2+} on neuraminidase activity of *B. fragilis* NCTC9344. An inoculum of 2 ml of a 48–h CMB culture was added to 200 ml of PPW5 broth and incubated anaerobically at 37°C for 48 h. After centrifugation, the packed cell deposit was resuspended in 5 ml of sterile normal saline and disrupted by ultrasonic vibration; the uncentrifuged cell extract was stored at −20°C.

Assay for neuraminidase activity. Human glycoprotein substrate FVII (Fraser and Smith, 1975) was used at a final concentration of 140–170 μg N-acetyl neuraminic acid (NANA)/ml reaction mixture (Fraser, 1978).

The standard assay procedures used by Fraser (1978) were followed. Tests for neuraminidase activity were first incubated for 60 min before assay for free NANA; when the 60–min assay showed little or no activity, tests were incubated for 24 h to demonstrate the presence or absence of detectable neuraminidase. Test spectrophotometric readings (E_{549}) were corrected by subtraction of the sum of the values for separate enzyme and substrate controls.

The corrected results of neuraminidase assays were graded as follows: $+++$, high values in 60–min assays ($E_{549} > 0{\cdot}5$); $++$, moderate NANA release clearly demonstrable in 60–min assays ($E_{549} > 0{\cdot}1$); $+$, neuraminidase not demonstrable in 60–min assays but high values ($E_{549} > 0{\cdot}5$) in 24–h assays; $\pm$, low values ($E_{549} < 0{\cdot}5$ but $> 0{\cdot}05$) in 24–h assays and absorption peak clearly shown at 549 nm; $-$, no peak at 549 nm demonstrable in 24–h assays.

Tests that gave low values ($E_{549} < 0{\cdot}5$) in 24–h neuraminidase assays were routinely examined to confirm that the absorption peak was at 549 nm (Fraser, 1978) and at least one test from each neuraminidase-positive species was also examined in this manner, whether the assay values were high or low. Culture products that gave very low values or negative results in 24–h neuraminidase assays ($E_{549} < 0{\cdot}5$) were further examined to determine whether destruction of NANA by N-acetyl neuraminic acid-aldolase (NAN-aldolase, EC 4.1.3.3) might be interfering with the 24–h neuraminidase assay (Fraser, 1978).

Studies of the effect of pH on the neuraminidase of B. fragilis. Neuraminidase assays were performed in a range of sodium-acetate buffers as described by Fraser and Smith (1975). The reaction mixture of 1·0-ml volume consisted of: 0·1 ml of the bulk cell extract of *B. fragilis* NCTC9344 (see above) diluted 1 in 40 in distilled water; 0·65 ml of the appropriate acetate buffer; and 0·25 ml of substrate FVII diluted in distilled water to give a final concentration in the reaction mixture of 160 μg NANA/ml. Separate enzyme and substrate controls were prepared at each *p*H value. Tests and controls were incubated at 37°C before the assay for NANA.

The *p*H values shown in the figure are the initial values for the buffers added to the tests. Separate measurements were made of the initial *p*H values in sample reaction mixtures prepared with equivalent proportions but in 3–ml total volumes.

Effect of Ca^{2+} and EDTA on the neuraminidase of B. fragilis. Neuraminidase assays in the presence of added Ca^{2+} or EDTA were performed as described by Fraser (1978). The test enzyme preparation was the bulk cell extract of *B. fragilis* NCTC9344 (see above) diluted 1 in 40 in acetate buffer, *p*H 5·1. Samples of the enzyme were mixed with buffer containing Ca^{2+} or EDTA and held at 37°C for 30 min before addition of substrate FVII and the start of the neuraminidase assay. Separate enzyme and substrate control tubes also contained Ca^{2+} or EDTA.

RESULTS

Production of neuraminidase by Bacteroides fragilis NCTC9344

Table I records the production of neuraminidase by *B. fragilis* strain NCTC9344 in 48–h cultures in various fluid media. The culture supernates contained small amounts of enzyme activity that could be detected when the incubation time of the assays was prolonged to 24 h. Cell extracts prepared from the same cultures contained larger amounts of neuraminidase that gave high assay values (E_{549}) in 60–min assays. Neither the culture supernates nor the cell extracts had significant NAN-aldolase activity that might interfere with the assay for NANA released by neuraminidase.

The organism grew adequately in all the media tested. Growth was least good in TGB and there were difficulties in centrifuging this culture because it contains a low concentration of agar. DB, PPW5 and BM broth media were chosen for further studies of neuraminidase production by *Bacteroides* species.

Effect of pH and Ca^{2+} on neuraminidase of B. fragilis NCTC9344

A more concentrated bulk cell extract was prepared from a 200–ml PPW5 culture of *B. fragilis* strain NCTC9344 for studies of the effect of *p*H and Ca^{2+} on the enzyme. The figure shows the results of 30–min neuraminidase assays in acetate buffers at different *p*H values. The assay values were corrected by subtraction of the values obtained in separate substrate and enzyme controls at each *p*H value; the enzyme controls were constant but the substrate controls were higher at low *p*H values.

The figure shows the *p*H optimum for *B. fragilis* neuraminidase with substrate FVII to be about 4·2. Acetate buffer at *p*H 5·1 is used in the standard assay (Fraser, 1978); this *p*H allows good neuraminidase activity and this

TABLE I

Production of neuraminidase by Bacteroides fragilis NCTC9344 in various culture media

Culture medium*	Degree of growth†	Relative amount of neuraminidase activity in culture product‡: culture supernate	cell extract
DB	+++	±	++
PPW5	++	±	+++
BM	++	±	+++
CMB	+++	±	+++
PPY	++	±	+++
TGB	+	+	+++
THB	+++	±	+++

* DB = Digest broth; PPW5 = proteose peptone water; BM = BM medium; CMB = cooked-meat broth; PPY = PPY medium; TGB = thioglycollate broth; THB = Todd–Hewitt broth.

† See *Methods* for assessment of growth.

‡ Supernates of 48-h cultures tested in 24-h assays; cell extracts from the same cultures tested in 60–min assays. See *methods* for grading of neuraminidase activity

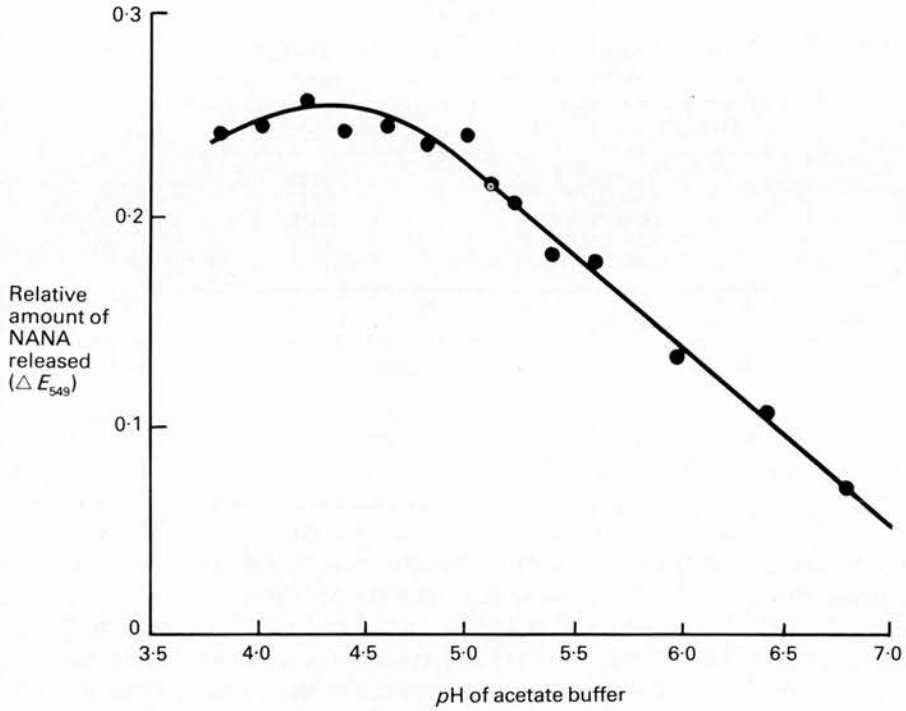

FIG.—Effect of *p*H on the assay for the neuraminidase of *B. fragilis*. Cell extract of *B. fragilis* strain NCTC9344 incubated with substrate FVII in sodium-acetate buffers.

buffer at this *p*H was therefore used in our tests for neuraminidase production by other *Bacteroidaceae*. Direct measurement of *p*H in equivalent test mixtures showed that the actual *p*H values might be 0·2–0·3 units higher, but they did not vary during incubation of the tests; thus the *p*H optimum for *B. fragilis* neuraminidase is likely to be about 4·5.

Table II shows that the addition of Ca^{2+} did not affect the assay for *B. fragilis* neuraminidase. The addition of 1 mM EDTA did not inhibit the enzyme and even 5 mM EDTA reduced the assay value by about one third only. Ca^{2+} was not added as a routine to our assays for neuraminidase production by *Bacteroides* strains.

Production of neuraminidase by species of the Bacteroides fragilis group

Table III shows the results of measuring neuraminidase produced by various species of the *B. fragilis* group. The strains were grown for 48 h in DB and PPW5 broths and cell extracts were tested for neuraminidase activity. In general these organisms grew better in DB than PPW5 broth but neuraminidase-positive strains usually produced greater activity in PPW5. All the strains of *B. fragilis* and *B. vulgatus* produced large amounts of neuraminidase in both media, and the strains of *B. distasonis* and *B. ovatus* were also clearly positive.

TABLE II

Effect of Ca^{2+} and EDTA on the assay of neuraminidase from B. fragilis NCTC9344

Concentration of added Ca^{2+} or EDTA in reaction mixture*	Result of neuraminidase assay (E_{549})†
3mM Ca^{2+}	0·186
1mM Ca^{2+}	0·171
None added	0·188
1mM EDTA	0·171
5mM EDTA	0·118

* See *Methods* for reaction mixtures.

† The spectrophotometric value (E_{549}) is an average result obtained from duplicate 15–min assay readings corrected by subtraction of the values for the corresponding substrate and enzyme controls.

All strains of *B. thetaiotaomicron* produced neuraminidase well in PPW5 but production in DB varied. Strain NCTC10582 was cultured in DB on four occasions; neuraminidase assays were twice positive (+ and + +) and twice negative. Variable results were also obtained with strain GNAB16 which was once positive (+) and twice negative. By contrast, three separate cultures of each organism in PPW5 broth were consistently positive.

Results with *B. variabilis* also varied. Strain GNAB18 consistently produced neuraminidase. Strain VPI11368 twice produced moderate amounts (+ +) in PPW5 but failed, however, to produce detectable activity in one other PPW5 culture and in three cultures in DB medium.

All the strains of *B. uniformis*, *B. eggerthii* and *B. splanchnicus* grew adequately but did not produce detectable neuraminidase in either medium.

Neuraminidase production by other Bacteroides species

The results of tests with other *Bacteroides* species are shown in table IV. All strains grew adequately in DB and most also grew well in BM medium. Many of these strains grew very poorly or not at all in PPW5 broth and so were not tested for neuraminidase production in this medium.

The three strains of *B. melaninogenicus* ss. *melaninogenicus* and the single strain of *B. melaninogenicus* ss. *levii* produced neuraminidase. Production by strain WPH67 varied; it twice gave + + activity in DB medium but was negative on another occasion and was twice negative in BM medium. The other strains were consistently positive in both media. By contrast the five strains of *B. melaninogenicus* ss. *intermedius* were negative in both media.

The strains of *B. oralis* and *B. bivius* were neuraminidase positive while *B. ruminicola*, *B. disiens*, *B. asaccharolyticus* and *B. corrodens* were all negative in both media. The *B. corrodens* strains did not grow well in BM broth; they were also grown and found to be negative in PPW5 medium.

Four strains of *B. ochraceus* produced neuraminidase well in DB, but production was very unreliable in BM and PPW5 media. Strain 79B produced trace amounts on three occasions in DB and once in BM broth. Strain VP12845 produced a trace of activity in only one of three cultures in DB and

TABLE III

Production of neuraminidase by organisms of the Bacteroides fragilis group grown in broth media

Test organism: strain no.	Growth* in		Relative amount of neuraminidase activity in culture product†	
	DB	PPW5	DB	PPW5
B. fragilis				
NCTC9343	+++	+++	+	+++
NCTC9344	+++	+++	+++	+++
WPH1	+++	+++	+++	+++
WPH12	+++	+++	+++	+++
WPH21	+++	+++	+++	+++
B. vulgatus				
NCTC10583	+++	+++	+++	+++
GNAB9	+++	+++	++	+++
GNAB29	+++	+++	++	+++
B. distasonis				
ATCC8503	+	++	+	++
GNAB26	++	+	++	+++
GNAB39	++	+	++	+++
B. ovatus				
ATCC8483	+++	+++	+	++
WPH207	+++	+++	±	++
B. thetaiotaomicron				
NCTC10582	+++	++	V	+++‡
GNAB1	+++	++	++	+++
GNAB2	+++	++	++	+++
GNAB7	+++	++	±	+++
GNAB11	+++	++	++	+++
GNAB16	++	+	V	++‡
GNAB20	+++	++	++	+++
B. variabilis				
VPI11368	+++	++	−‡	V
GNAB18	+++	++	+‡	+++‡
B. uniformis				
ATCC8492	+++	+++	−	−
VPI11227	+++	++	−	−
GNAB42	+++	+++	−	−
B. eggerthii				
NCTC11155	++	+	−	−
GNAB21	+++	+	−	−
GNAB23	+++	++	−	−
GNAB24	++	+	−	−
GNAB41	++	+	−	−
GNAB43	+++	++	−	−
B. splanchnicus				
NCTC10825	+++	+++	−	−
NCTC10826	+++	+++	−	−

* See *Methods* for assessment of growth. DB = Digest broth; PPW5 = Proteose peptone water.

† Cell extract from 48-h culture in stated medium was tested. See *Methods* for grading of neuraminidase activity. V = variable results in different experiments (see text).

‡ Result confirmed in repeat culture.

TABLE IV

Production of neuraminidase by strains of various Bacteroides species grown in broth media

Test organism: strain no.	Growth* in		Relative amount of neuraminidase activity in culture product†	
	DB	BM	DB	BM
B. melaninogenicus ss. melaninogenicus				
ATCC15930	++	++	++	+++
VPI4196	++	+++	±	++
WPH67	+++	+++	V	−*
B. melaninogenicus ss. levii				
VPI3300	+++	+++	+++	+++
B. melaninogenicus ss. intermedius				
NCTC9336	+++	++	−	−
NCTC9338	++	+	−	−
WPH4	+++	+++	−	−
WPH26	+++	++	−	−
WPH31	++	++	−	−
B. oralis				
VPI8906D	++	++	++	++
VPI9958	++	...	+++	...
GNAB46	++	...	+	...
GNAB54	+	...	+	...
B. bivius				
VPI5540	+	++	+	++
VPI6318	+	...	+	...
VPI6822	++	...	++	...
VPI7880	++	...	++	...
B. ruminicola				
NP333	++	++	−	−
B38024	+++	+	−	−
B38080	+	+	−	−
B56029	+	+	−	−
B. disiens				
VPI7852	+	+++	−	−
VPI8057	+++	+++	−	−
B. asaccharolyticus				
NCTC9337	++	++	−	−
WPH15	+	++	−	−
WPH30	++	++	−	−
WPH57	++	+	−	−
B. corrodens				
143A	+	±	−	−
151RV	+	±	−	−
B. ochraceus				
VPI2845	++	+	V	−‡
10	++	+	++	−
73	++	++	++	±
79B	+++	+	±‡	V
1956C	+++	+	+++	−
2467B	+++	++	++	−

* See *Methods* for assessment of growth. . . . = not tested; DB = digest broth; BM = BM broth.

† Cell extract from culture in stated medium was tested. See *Methods* for grading of neuraminidase activity; . . . = not tested. V = variable results in different experiments (see text).

‡ Result confirmed in repeat culture.

was consistently negative in BM and PPW5 cultures. The difficulty in demonstrating neuraminidase activity in these cultures may be partly explained by the presence of small amounts of NANA-destroying activity in the cell extract of several of the *B. ochraceus* cultures.

Production of neuraminidase by Fusobacterium and Leptotrichia species

Table V shows the results when eight strains of *Fusobacterium* species and one of *Leptotrichia buccalis* were grown in DB and PPW5 media. In general they grew well in DB and less well in PPW5. The two strains that grew poorly in PPW5 were also grown and tested in BM medium. Cell extracts of all cultures were neuraminidase negative.

TABLE V

Tests for production of neuraminidase by Fusobacterium and Leptotrichia species grown in broth media

Test organism: strain no.	Growth* in		Relative amount of neuraminidase activity in culture product†	
	DB	PPW5	DB	PPW5
F. necrophorum				
NCTC10575	+++	++	−	−
NCTC10576	++	+	−	−
NCTC10577	++	+	−	−
F. polymorphum (F. nucleatum)				
NCTC10562	+++	+	−	−
WPH142	++	+	−	−
WPH160	++	±	−	...
F. necrogenes				
NCTC10723	+	+	−	−
F. varium				
NCTC10560	++	+	−	−
L. buccalis				
NCTC10249	++	−	−	...

* See *Methods* for assessment of growth. DB = digest broth; PPW5 = proteose peptone water.

† Cell extract from culture in stated medium was tested. See *Methods* for grading of neuraminidase activity. ... = Not tested.

DISCUSSION

Neuraminidase is produced as an extracellular enzyme in culture supernates by *Vibrio cholerae* and *Clostridium perfringens*, but is cell-associated in other species, e.g., *Klebsiella aerogenes* and *Pasteurella multocida* (see Rosenberg and Schengrund, 1976). Our previous studies confirmed that clostridial neuraminidases are essentially extracellular, although some more enzyme could be released by ultrasonic disintegration of the cells (Fraser and Collee, 1975; Fraser, 1978). By contrast, the results presented in table I show that the

neuraminidase of *B. fragilis* is predominantly cell associated, with only small amounts detectable in the supernates of 48–h cultures.

We have adapted our clostridial neuraminidase-assay techniques for use with cell extracts of *B. fragilis* and other Bacteroidaceae. The *p*H optimum for neuraminidases may vary considerably with different buffer systems and different substrates (Fraser and Smith, 1975; Rosenberg and Schengrund, 1976) but our standard assay with human glycoprotein substrate FVII with acetate buffer at *p*H 5·1 also gave good results with the *B. fragilis* enzyme (see the figure). The neuraminidase produced by certain species, e.g., *V. cholerae*, is calcium dependent but added Ca^{2+} is not required in our assays for the enzyme produced by clostridia (Fraser, 1978) or *Bacteroides* species (table II).

Many of the Bacteroidaceae are nutritionally fastidious and do not generally grow well in fluid media even with growth supplements and meticulous anaerobic technique. The three media used in the present study were chosen for their ability to support good enzyme production by neuraminidase-positive species and also to allow good growth of a wide range of gram-negative anaerobes. DB medium allowed adequate growth of all species tested but did not always allow the best production of neuraminidase. Some of the *B. fragilis* group of organisms produced more enzyme in PPW5 broth; however, this medium did not support adequate growth of several other species, even when incubation was extended to 96 h. A few of these strains produced more enzyme in BM broth than in DB medium, but there were also clear exceptions. Neuraminidase-positive strains generally produced the enzyme in each of the two media in which they were grown, but production by a few varied or did not take place in one or other (see tables III and IV). The purity of all cultures was carefully tested by subculture from the test culture. Neuraminidase production has been shown to be inducible in certain species (Nees and Schauer, 1974; Rosenberg and Schengrund, 1976) but we have not been able to identify specific inducing conditions that might explain the variations observed in this study between different media or different batches of the same medium.

Because no single medium consistently supported optimum production by neuraminidase-positive strains, other strains were tested in at least two media that supported adequate growth. All cell extracts were tested in assays incubated for 24 h before being classed as neuraminidase negative.

The neuraminidase assay depends upon detection of NANA released from the glycoprotein substrate by the enzyme. Some organisms also produce an aldolase (NAN-aldolase) that may destroy the released NANA and this might give false negative results in neuraminidase assays. The NAN-aldolase of clostridia remains cell associated and does not interfere with assays for neuraminidase in culture supernates, although it may reduce the sensitivity of assays with cell extracts (Fraser and Collee, 1975). Müller and Werner (1970*b*), using a paper chromatographic method, reported the presence of NAN-aldolase in several species of *Bacteroides*, including *B. fragilis*, and Müller (1973) also found that a strain of *Fusobacterium polymorphum* produced the aldolase. We have not assayed directly for NAN-aldolase (Brunetti, Swanson and Roseman, 1963; Nees *et al.*, 1976) but we have shown that there is negligible NANA-

destroying activity in the cell extracts of the neuraminidase-positive *B. fragilis* NCTC9344 and in the cell extracts of the various cultures that gave low values or negative results in neuraminidase assays. Very few of these cell extracts reduced the assay value for added NANA by as much as 10–20% during 24-h incubation under conditions equivalent to those of the neuraminidase assay. This amount of NANA destruction would not appreciably reduce the ability of the assay to detect small amounts of neuraminidase. Several of the *B. ochraceus* cell extracts reduced the added NANA by about 40% in 24 h and this could have contributed to our difficulty in demonstrating neuraminidase production consistently in this group.

The classification of the Bacteroidaceae has been much refined in the past decade. Current schemes of classification are based mainly on the biochemical activities of the organisms (Holdeman and Moore, 1974; Holdeman, Cato and Moore, 1977). The strains used in the present study have been characterised carefully by the methods used in this laboratory, including gas–liquid chromatography (Duerden *et al.*, 1976; Deacon *et al.*, 1978; Duerden *et al.*, 1980). The former subspecies of *B. fragilis* are now given separate species status, and *B. thetaiotaomicron* is further subdivided into the species *B. thetaiotaomicron*, *B. eggerthii*, and *B. uniformis*. Strain ATCC8492, originally the reference strain of *B. thetaiotaomicron*, is now classified as *B. uniformis*. Werner (1974) demonstrated that sugar fermentation tests discriminated reliably between the various species in this group; he further distinguished *B. variabilis* from *B. uniformis* by differences in fermentation of rhamnose and trehalose.

The classification of the *B. oralis–B. melaninogenicus* group has been considerably revised in recent years, with less emphasis placed on pigment production as the criterion for classifying strains as *B. melaninogenicus*. Because of the basic difference in sugar utilisation, *B. melaninogenicus* ss. *asaccharolyticus* has been given separate species status as *B. asaccharolyticus*, while *B. melaninogenicus* is now divided into the subspecies *melaninogenicus*, *levii* and *intermedius*. There has been some debate about the classification of *B. oralis* strains. Strain ATCC15930, originally a reference strain of *B. oralis*, is now reclassified as *B. melaninogenicus* ss. *melaninogenicus* (Holbrook and Duerden, 1974). Strain NP333, previously described as *B. oralis*, is now reallocated to *B. ruminicola*. Strains of *B. ochraceus* differ from other Bacteroidaceae in that they require CO_2 for growth but are not strict anaerobes (Holbrook, Duerden and Deacon, 1977). It has recently been proposed that they should be reclassified in the new genus *Capnocytophaga* (Newman *et al.*, 1979; Williams, Hollis and Holdeman, 1979).

Our results for neuraminidase production by *Bacteroides* are consistent within each species and tend to confirm the present classification. Within the *B. fragilis* group, *B. fragilis*, *B. vulgatus*, *B. distasonis* and *B. ovatus* were all neuraminidase positive. Seven strains of *B. thetaiotaomicron* were positive while six strains of the closely related *B. eggerthii* were negative. The *B. variabilis* strains were distinguished from the *B. uniformis* strains by their production of the enzyme. Within the *B. oralis–B. melaninogenicus* group, *B. oralis* and *B. melaninogenicus* ss. *melaninogenicus* were neuraminidase positive

while ss. *intermedius* and *B. ruminicola* were negative; the single strain of *B. melaninogenicus* ss. *levii* tested was neuraminidase positive. *B. bivius* produced the enzyme, while the closely related *B. disiens* did not. The asaccharolytic species, *B. asaccharolyticus* and *B. corrodens*, were both negative. The six strains of *B. ochraceus* tested could all produce neuraminidase although it was difficult to define conditions allowing reliable production.

Production of neuraminidase by *Bacteroides* species was studied by Müller and Werner (1970*b*; Werner and Müller, 1971). These studies depended primarily on observing the electrophoretic changes in serum proteins in the culture medium after incubation for 2–19 days, but were also supported by paper chromatography of neuramin-lactose and its split products. They found that all of 24 strains of *B. fragilis* produced the enzyme, but only six of 11 *B. vulgatus*, three of five *B. distasonis* and six of eight *B. thetaiotaomicron*. With our assay procedures, testing cell extracts of the cultures, all the strains of these species examined were neuraminidase positive. At least one of their negative *B. thetaiotaomicron* strains (ATCC8492) is now reallocated to *B. uniformis*; this strain was negative in our assays also. Their findings with other *Bacteroides* species are in broad agreement with our present results: *B. ovatus* ATCC8483, three strains of *B. oralis* (including ATCC15930) and one strain of *Leptotrichia innominata* var. *ochracea (B. ochraceus)* were positive, and single strains of *B. asaccharolyticus* (NCTC9337) and of *B. putredinis* were negative.

Müller and Werner (1970*a*) also studied pus from a *B. fragilis* abscess and showed that neuraminidase was produced *in vivo*. They suggested that the neuraminidase might play a role in the pathogenicity of *B. fragilis*, demonstrating that it is produced in greater amounts and has a broader spectrum of action against serum glycoproteins than the enzyme produced by other *Bacteroides* species. *B. fragilis* is now an accepted pathogen; although it is only a minor component of the anaerobic gut or vaginal flora it is much the most common *Bacteroides* species isolated from clinical infections (Duerden, 1980*a* and *b*). The pathogenic potential of other *Bacteroides* species is much less clearly understood at present; it will be interesting to note whether there is any correlation with their ability to produce neuraminidase.

Fukui, Fukui and Moriyama (1971) failed to detect neuraminidase in four strains of *Fusobacterium* species and two of *Leptotrichia buccalis* isolated from the mouth, nor could we detect the enzyme in cell extracts of the strains that we examined. However, Werner and Müller (1971) detected weak neuraminidase activity against plasma proteins in two of 10 strains of *Sphaerophorus necrophorum (F. necrophorum)*, two of three *S. varium (F. varium)* including NCTC10560, and one of seven other *Fusobacterium* strains. Müller (1973) isolated a strain of *F. polymorphum* from the throat of a patient with actinomycosis; it showed strong neuraminidase activity when first isolated but this declined to a low level during subsequent laboratory passage. Müller (1973, 1974) has argued that neuraminidase plays a role in the pathogenicity of various organisms and that in some cases its production may be induced *in vivo*. The *Fusobacterium* strains that we found to be neuraminidase negative have been maintained in laboratory culture for some years and it remains

possible that they have lost the ability to produce the enzyme during storage.

Solovev *et al.* (1972) found tests for neuraminidase production of value for studies of vibrio taxonomy and our previous studies suggested that they might also help to differentiate some closely related clostridia, e.g., *C. sordellii* and *C. bifermentans* (Fraser, 1978). The taxonomy of the fusobacteria is still being debated (see Duerden *et al.*, 1980) and our failure to demonstrate neuraminidase in any of the strains tested is unlikely to be of assistance. However, the results of our tests in *Bacteroides* species are consistent and conform with the latest classifications (Holdeman *et al.*, 1977; Duerden *et al.*, 1980). They help to discriminate between several closely related species that are otherwise distinguished only by a small number of sugar fermentation reactions, e.g., between the species previously included in *B. thetaiotaomicron*, and between various species of the *B. oralis–B. melaninogenicus* group. Tests for neuraminidase production would therefore be a valuable addition to the range of biochemical tests currently used in classification of *Bacteroides* organisms.

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Current Approaches to the Classification, Characterisation and Typing of Pathogenic Anaerobic Bacteria

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ABSTRACT. Characterisation systems based on the demonstration of soluble antigens and toxins are of much practical value but have clear taxonomic limitations. This is illustrated by reference to our work with some of the pathogenic clostridia. The definition and classification of the anaerobic cocci is a difficult and continuing challenge. Systems for the classification and characterisation of the gram-negative non-sporing anaerobic bacilli are evolving well, though there are still some very difficult areas. A selection of the most helpful tests is indicated. Biotyping systems are compared with serotyping systems; the results of serological approaches to characterisation are encouraging. The further exploration of bacteriocin typing seems to be merited. The challenge of characterisation and classification of anaerobic spirochaetes commonly involved in human infections must now be taken up.

INTRODUCTION

So much work is being done on the anaerobic bacteria that it is helpful to consider practical aspects of classification and characterisation in 1981, to update our views expressed in 1979 (1). In this paper, we review significant trends and we consider results of our recent studies of various pathogenic anaerobes, with special reference to practical approaches to their prompt characterisation and effective classification in relation to medical and dental bacteriology.

MATERIALS AND METHODS

Organisms: the strains studied are detailed in recent papers published by past and present members of our research team; the relevant references are given in the text. Biochemical tests were as described previously (5, 6). Gas liquid chromatography: The procedures used were described by Deacon and colleagues (4). Neuraminidase assays were performed as described by Fraser and Brown (8, 9). Bacteriocin studies were as outlined by Watt and Collee (22).

RESULTS AND DISCUSSION

The clostridia. Two organisms provide interesting examples of challenges to our existing systems of classification and characterisation. These are *Clostridium perfringens* and *C. difficile*.

C. perfringens. Although strains of types Al and A2 replicate very rapidly at 44–45°C with mean generation times of less than 10 min (2, 3) we found that production of lecithinase (phospholipase-C) is suppressed at these temperatures. As detection of this enzyme is important in current primary identification tests, the organism's potential for very rapid growth has not been exploited significantly in the diagnostic laboratory.

C. perfringens-like organisms, including *C. absonum, C. paraperfringens* and *C. sardiniensis*, differ in relatively minor characteristics from typical *C. perfringens* (14, 15). It is important to keep in touch with developments in this area.

Any approach that might help to sub-divide the *C. perfringens* complex merits exploration as our present dependence on animals for toxin neutralisation tests in the typing of the organism is a great disadvantage. The use of agglutination tests for the typing of strains involved in food-poisoning has been very helpful (10).

C. difficile. Our experience with this organism has recently been reviewed by Poxton (17). We characterise the organism on the basis of its microscopic morphology, its ability to grow on CCFA medium, its performance in biochemical tests, the characteristic profile of end-products detected by gas-liquid chromatography (GLC), and the detection of characteristic antigens in crossed immunoelectrophoresis systems when tested against rabbit antiserum raised against whole cells of the type strain (NCTC 11223). Antigenic cross-reactions with the *C. sordelli/bifermentans* complex can be

absorbed out. Some strains do not produce the typical cytotoxin and it is clear that more work is needed on the debated role of the cytotoxin in the enteropathogenicity of this organism. Some of our strains showed minor variations in the presently accepted range of biochemical tests used for characterisation; several showed significant differences, and two strains showed marked departures from the recognised pattern for *C. difficile* published in the VPI manual (12). Differences of this degree would certainly qualify for separate species rank in the *Bacteroides* genus (see below). We are anxious to determine whether the variation offers a basis for biotyping which may be of epidemiological value in tracing infective strains.

Neuraminidase production as an aid to clostridial taxonomy

The possible association of neuraminidase production with pathogenic potential is debated, but the enzyme may be of taxonomic use (8). Table I lists the neuraminidase-positive and neuraminidase-negative clostridia. This appears to distinguish clearly between *C. sordelli* and *C. bifermentans* and complements the case for regarding these as separate species.

Amino acid utilization and production in relation to taxonomy

Studies to explore the metabolism of amino acids by clostridia as an aid to our understanding of the proteolytic group deserve our attention (7).

Bacteroides-Fusobacterium group. Presumptive identification at genus level can be made by observations on Gram smears, colonial morphology and a few other tests. GLC analysis of short chain fatty acid end-products of metabolism can also identify to genus level, but further tests are required to establish the species or sub-species of *Bacteroides* and *Fusobacterium* (4). We select the observations and tests indicated in Table II as the currently recommended range (4, 5, 6, 11).

The experience of others (12, 13) encourages us to replace mannitol with salicin, and we note promising results with xylan (19). The range of organisms indicated in Table II is not complete and is intentionally restricted to those species that might merit specific recognition in the clinical laboratory. Dental bacteriologists may soon press for the inclusion of *Bacteroides gingivalis* as an important species. The restriction to genus level for *Fusobacterium* species is unwise and merely reflects our present lack of extensive experience with this group.

We now restrict the tolerance tests to taurocholate and to one dye only, in view of practical difficulties with Victoria blue. The two tests give information that helps significantly to compensate in a laboratory that might not be able to afford the expense of a GLC system.

Serological characterisation. In studies that exploit the species-specific antigenicity of the cell surface outer membrane (OM) complex and the sensitivity of an indirect enzyme-linked immunosorbent assay (ELISA), we are now able to identify many *Bacteroides* strains to species and sub-species level (16, 18). Table III summarises results obtained with OM preparations from representative reference laboratory and freshly isolated strains with species reference antisera that we prepared against whole cells and tested in an indirect ELISA system. The outer membrane preparations are obtained by treatment of bacterial suspensions with 10 mM EDTA at 45° C for 30 min and then holding in an ultrasonic bath for 60 s. The antisera are raised in rabbits against whole live bacterial suspensions. Heterologous cross-reacting antibody can be removed by absorption of the antiserum with whole cells. We find very significant correlation between serotype and biotype in this work. Results were clear-cut with the *B. fragilis* complex, the asaccharolytic group, and with *B. melaninogenicus intermedius*. The results with the *B. melaninogenicus-ruminicola* complex reflect the confusion that also afflicts conventional biotyping systems.

Table I. *Neuraminidase studies with clostridia*

Neuraminidase-positive clostridia
C. perfringens, types A–E
C. septicum
C. chauvoei
C. tertium
C. sordelli
C. absonum
Neuraminidase-negative clostridia
C. novyi, types A–D
C. tetani
C. botulinum
C. sporogenes
C. bifermentans
C. histolyticum
C. paraperfringens
C. butyricum
C. sphenoides
C. fallax
C. tetanomorphum=cochlearium
C. subterminale
C. difficile

Table II. *A practical approach to the characterisation of commonly encountered gram-negative non-sporing anaerobic bacilli of clinical interest*

+ = > 95% of strains give positive result, − = > 95% of strains give negative result, v = variation 30−70%, +(−) = positive with most strains, −(+) = negative with most strains. For details see Duerden, Holbrook, Collee & Watt, 1976. J appl Bacteriol 40: 163−188. The characterization of clinically important gram-negative anaerobic bacilli by conventional bacteriological tests.

	B. fragilis	*B. vulgatus*	*B. distasonis*	*B. thio.*	*B. ovatus*	*B. mel. intermed.*	*B. mel. mel.*	*B. oralis*	*B. ruminicola*	*B. bivius*	*B. disiens*	*B. asaccharolyticus*	*B. ureolyticus*	*Fusobacterium* spp.[a]
Pigment	−	−	−	−	−	+	+	−	−	−	−	+	−	−
Indole	−	−	−	+	+	+	−	−	−	−	−	+(−)	−	v
Aesculin hydrolysis	+	v	+	+	+	−	v	+(−)	+	−	−	−	−	−(+)
Fermentation of														
Glucose	+	+	+	+	+	+	+	+	+	+	+	−	−	v
Lactose	+	+	+	+	+	−(+)	+	+	+	+	−	−	−	−(+)
Sucrose	+	+	+	+	+	+	+	+	+	−	−	−	−	−(+)
Rhamnose	−	+	+(−)	+	+	−	−(+)	v	+(−)	−	−	−	−	−
Trehalose	−	−	+	+	+	−	−	−	−	−	−	−	−	−(+)
Xylose	+(−)	+	+	+	+	−	−	−	+	−	−	−	−	−
Salicin	−	−(+)	+	−(+)	+	−	−	−(+)	+(−)	−	−	−	−	−(+)
Significant FA Produced[b]														
Succinic			++					++				(+)	+	−(+)
Acetic			++					++				++	+	+
N-Butyric			−					−				++	−	++
Tolerance of[c]														
Sod. Taurocholate			+					−				−	−	v
Gentian Violet			−					−				−	−	+
Resistance to[d]														
Neomycin 1000 μg			R					S				S/R	S	S
Kanamycin 1000 μg			R					R				R	S	S
Penicillin 2 units			R					S/R				S	S	S
Rifampcin 15 μg			S					S				S	S/R	S/R

[a] Alternative reactions in this column relate to species differences as well as strain variations.
[b] GLC: + = 1−10 μmol/ml, ++ = > 10 μmol/ml, (+) = variable amount of minor product.
[c] Tolerance: Taurocholate 0.5%, Gentian violet 1 in 100 000, + = tolerant, − = inhibited.
[d] Antibiotic resistance: R = resistant, S = sensitive, S/R = variation (30−70%).

Neuraminidase studies. Table IV lists the neuraminidase-negative and positive *Bacteroides* species and may help in classifying the *melaninogenicus-ruminicola-oralis* complex and the *bivius-disiens* group. The few *Fusobacterium* species that we have tested do not produce neuraminidase (9). Our neuraminidase tests helped to discriminate between closely related *Bacteroides* species that are presently differentiated by a small number of sugar fermentation reactions. As the results are consistent for all strains of each of the species that we examined, the findings might be of taxonomic value (9). In view of the technical difficulties of neuraminidase assays, it will be necessary to develop a simple test; for example, the enzyme's ability to confer panagglutinability on human red cells might be exploited.

Bacteriocin typing. Bacteriocin-like substances produced by *Bacteroides* species offer a possible approach to typing and sub-classification (22). It was first necessary to develop detailed characterisation studies and now it seems worthwhile to re-examine the "bacteroidicins" in which we were interested some years ago, but now to recognise them as fragilicins, etc. It has been possible to analyse our earlier results in the light of our subsequent characterisation of the organisms that we tested

Table III. *Results of serological studies with outer-membrane antigens and homologous antisera in an indirect ELISA system*

Species and strain(s) used as antigen	Autologous titre[a] (T)	Number of homologous strains yielding preparations that reacted to at least T/8 (out of number tested)
B. fragilis		
NCTC9344	12 800	34 (35)
B. vulgatus		
NCTC10583	25 600	11 (11)
B. distasonis		
ATCC8503	12 800[a]	
GNAB26	12 800	6 (8)
B. thio.		
NCTC10582	25 600[a]	
GNAB11	3 200	8 (10)
B. mel. intermed.		
NCTC9338	12 800	11 (11)
B. mel. mel.		
ATCC15930	12 800[a]	
VPI4196	25 600	6 (11)
B. oralis		
1210	51 200	5 (17)
B. ruminicola		
NP333	25 600[a]	
GA33	6 400	6 (14)
B. asaccharolyticus		
NCTC9337	25 600	24 (25)

[a] The autologous titre denotes the titre obtained with the strain used as antigen. When two antisera were prepared for a species, the combined results are given.

Table IV. *Results of neuraminidase studies with organisms of the Bacteroides-Fusobacterium group*

Neuraminidase-positive Bacteroides spp.	
B. fragilis	*B. mel. ss. melaninogenicus*
B. vulgatus	*B. mel. ss. levii*
B. distasonis	*B. oralis*
B. ovatus	*B. bivius*
B. thetaiotaomicron	
B. variabilis	
(B. ochraceus = Capnocytophaga)	
Neuraminidase-negative Bacteroides spp.	
B. eggerthii	*B. mel. ss. intermedius*
B. uniformis	*B. ruminicola*
B. splanchnicus	*B. disiens*
	B. asaccharolyticus
	B. corrodens
Neuraminidase-negative fusobacteria	
F. necrophorum	*L. buccalis*
F. polymorphum	
F. varium	
F. necrogenes	

(Table V). The indications are that most strains of *B. fragilis* are both good producers and indicators, whereas strains of *B. thetaiotaomicron* studied by us were poor in this respect. Our experience with the *B. melaninogenicus* group is presently inadequate. The results obtained with the *B. asaccharolyticus* strains seem to be promising, as the producers showed their effect only against *B. asaccharolyticus* indicators. These are retrospective analyses and a note of caution is needed; the agents responsible for these effects have not been formally characterised as bacteriocins and a systematic study is now indicated with a range of characterised strains.

The anaerobic cocci

We certainly confirm that "the clinical microbiologist attempting to isolate and characterise the obligately anaerobic cocci, and the clinician attempting to treat infections caused by these organisms, both suffer from a lack of useful data" (24). We accept the provisional definition of Watt and Jack (23) that anaerobic cocci grow under satisfactory conditions of anaerobiosis and do not grow on suitable solid media in 10% CO_2 in air even after incubation for 7 days at 37° C. These workers found that metronidazole sensitivity is a reliable index; obligately anaerobic cocci are all sensitive to metronidazole.

The detailed descriptions provided by Smith and Holdeman (20) provide a helpful starting point for a review of classification of this difficult group. We find the inclusion of *Streptococcus* confusing. The division of the streptococci into truly anaerobic and facultative groups might be taxonomically proper, but facultative strains are encountered in clinical specimens that cannot be isolated initially unless

Table V. *Results of preliminary studies of bacteriocin-like effects with gram-negative anaerobic bacilli*

Species	Producers		Indicators	
	Number Positive	Number Tested	Number Positive	Number Tested
B. fragilis	34	37	28	34
B. thio.	1	6	1	6
B. vulgatus	0	5	4	4
B. distasonis	0	1	0	1
B. mel. ss. intermed.	2	2	0	2
B. mel. ss. mel.	1	1	1	1
B. asaccharolyticus	4	4	4	4
B. ruminicola	0	1	0	1
Fusobacterium spp.	2	5	0	3

naerobic culture procedures are undertaken (20). his may be explained on the basis of carbon dioxle dependence and other exacting considerations iat merit further work. Thus, apparently anaerobic occi that are not true anaerobes still feature in our ollections, and sometimes in our clinical reports. he anaerobic state and sensitivity to metronidaole is of clinical importance, but it is inevitable that ie rules of convenience must bow to the stricter ules of a system that might well include obligate naerobes and facultative organisms in one genus. ccordingly, the clinical bacteriologist must be on uard; we make the lighthearted but significant omment that some of our organisms at the primary olation stage have not read the textbook.

he anaerobic spirochaetes

Ve are again indebted to Smith and Holdeman (20) or a helpful outline. In addition to the well-known uman pathogenic associations of the anaerobic pecies in the genera *Spirochaeta*, *Borrelia* and *Treonema*, the association of spirochaetes with other rganisms in periodontal disease is of much interst. The occurrence of spirochaetes in the human olon, especially noted in male homosexuals, also nerits further study. Smith takes the view that it is oubtful whether any of the cultivable treponemes re significantly pathogenic in the mouth. The inrease in number of these organisms in areas of ingival pathology has been recognised for years, ut the correlation of these organisms with degrees f tissue destruction has stimulated interest among ental bacteriologists and dental practitioners conerned with periodontal disease (21). There is athogenic synergy in these infections and, while it nay be that the spirochaetes are not a significant omponent of the initial microbial attack and may e merely secondary opportunists exploiting condiions that encourage their growth, many of us have een slow to develop adequate investigations of eriodontal disease. It is likely that the significant rimary pathogens will include bacteroides organms and fusobacteria and perhaps *Capnocytohaga*-like organisms which abound in these leions. Recent developments in the classification nd characterisation of these organisms should now llow us to make significant progress in this imporint field.

ONCLUSION

ur aim in this paper has been to consider the degrees of precision regarded as necessary and practical in the characterisation and classification of some representative anaerobic bacteria of clinical importance. It is paradoxical that the label of *C. perfringens* presently applies to a remarkably wide range of human and animal pathogens that can only be distinguished by difficult toxigenic analyses, whilst new species rank is afforded to relatively minor variant biotypes. In this case, serological analyses of soluble antigens have so far helped only in typing food-poisoning strains. On the other hand, *C. difficile* seems to embrace many biotypes; present confusion concerning the significance of the cytotoxin and the enterotoxin in its identification encourages us to develop somatic serological studies. Further studies of amino acid metabolism might also be helpful. The successful biotyping of the *Bacteroides-Fusobacterium* group has been extended to the point at which minor biochemical variants qualify for species rank, and this seems to be supported by serological analyses of species-specific outer membrane antigens. Other possible approaches include bacteriocin typing and this may have epidemiological value. In contrast, the classification of the anaerobic cocci is still poorly developed, and the oral spirochaetes merit more systematic study.

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